



Abstracts of the 13th World Congress on Alternatives and Animal Use in the Life Sciences, Rio de Janeiro, 2025

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ALTEx Proceedings

Octavio A. F. Presgrave
Welcome

Alternatives Congress Trust
Welcome



Human Health

Animal Health

Environmental Health

One Health

Education



Springer

**Octavio Presgrave****Chantra Eskes****Marize Valadares**

Dear friends, dear colleagues

We warmly welcome you to the 13th World Congress on Alternatives and Animal Use in the Life Sciences (WC13), which will take place from August 31st to September 4th, 2025, in Rio de Janeiro, Brazil.

We are very proud to host one of the largest congresses in this area in this vibrant location. After over 30 years since its establishment in 1993, for the first time the World Congress takes place in the southern hemisphere. Brazil has a large community working on the research, development and implementation of alternative methods and of the 3Rs principles in different areas. Just recently, on July 30th, 2025, Brazil adopted a law banning the use of experimental animals for cosmetics testing.

We are thrilled to offer a high-quality scientific programme covering five main topics including Human Health, Animal Health, Environmental Health, Education, and for the first time One Health. We hope you will be inspired by the 77 scientific sessions, 9 satellite events, 4 early investigators flash presentation sessions, and one early career session offered by the programme. The programme comprises six keynote lectures as well as 340 oral presentations and 368 poster presentations covering the five main topics of the congress and gathering participants from six different continents.

We are confident that the congress will offer you an excellent platform to network, share best practices, discuss the latest developments in the field, and collaborate to help accelerate progress towards reducing, refining, and replacing the use of animals in scientific research and testing. Rio de Janeiro, where nature meets progress, offers an inspiring scenario for such exchanges. We hope you can enjoy the diversity of its landscapes, its famous beaches, natural reserves, parks, mountains, as well as its historical and cultural heritage, including bossa nova, chourinho and samba music.

We take this opportunity to give our special thanks to our hosts, FioCruz and BraCVAM, to the Scientific Committee, the Local Organizing Committee and the Communication Committee, as well as to all sponsors, exhibitors, supporters and participants who have made this congress possible.

Looking forward to meeting you and to help shape the future direction of research and policy in this area, contributing to the promotion of more humane and scientifically sound approaches to advancing the life sciences.

Enjoy the 13th World Congress on Alternatives and Animal Use in the Life Sciences in Rio de Janeiro!

Octavio Presgrave, Chair of WC13

Also on behalf of Chantra Eskes and Marize Valadares, co-chairs of WC13



Elaine Faustman



Thomas Hartung



Gilly Griffin

Message from the Alternatives Congress Trust (ACT)

What a remarkable journey we're on together. Just two short years ago, we celebrated three decades of the World Congress series at WC12. Today, we make history again as we gather in Latin America for the very first time – a testament to the truly global reach of our movement.

The pace of change has been breathtaking. In just 24 months, we've witnessed groundbreaking advances in new approach methodologies and secured unprecedented governmental commitments to eliminate animal reliance in safety testing. Right here in Brazil, lawmakers have taken a bold stand, banning cosmetic and personal care testing on vertebrates. Across the Atlantic, the US National Institutes of Health has declared it will no longer call for animal-only research proposals and now will seek development of human-relevant models. US FDA, US EPA, Canada, and Europe announced roadmaps to reduce animal testing. These aren't just policy victories – they're seismic shifts that signal a new era.

Our theme, "3Rs Integrating 3 Worlds: Human, Animal and Environmental Health," captures something profound: The 3Rs aren't just laboratory practices – they're pillars of a healthier, more ethical world where human wellbeing, animal welfare, and environmental sustainability converge under the One Health, One Welfare umbrella.

Behind every breakthrough you will experience at WC13 lies the dedication of our Congress organizing and scientific committees, who have crafted a program as diverse as our global community. Whether you're a seasoned advocate returning to reconnect with old allies or a bright-eyed early career scientist taking your first steps into the 3Rs world, this Congress belongs to you.

Once again, *ALTEX Proceedings*, generously sponsored by the Doerenkamp-Zbinden Foundation, has produced this abstract book – your roadmap to the cutting-edge research, policy innovations, and ethical frameworks that are reshaping science itself. These pages don't just document progress; they illuminate the path to a future where scientific excellence and compassion walk hand in hand.

From all of us at the Alternatives Congress Trust, welcome home. You've traveled from every corner of the globe to be here, bringing your unique perspectives, groundbreaking research, and shared vision. Over the coming days, learn voraciously, connect authentically, and leave knowing that together, we're not just changing science – we're changing the world.

The future is bright, and it starts now.

Elaine Faustman, ACT Chair; Thomas Hartung, ACT Treasurer; and Gilly Griffin, ACT Secretary

**Dear WC13 participants,**

ALTEX Proceedings is proud to present the Abstract Book of the 13th World Congress on Alternatives and Animal Use in the Life Sciences in Rio de Janeiro, Brazil.

This Abstract Book contains summaries of 340 oral presentations and 368 posters. They are sorted by presentation type and submission ID number. The abstracts address the five Congress Themes: Human Health, Animal Health, Environmental Health, One Health, and Education. About 2200 authors from 46 countries on six continents have contributed their work.

Once again, we are very grateful to the Doerenkamp-Zbinden Foundation, Switzerland for generously funding the production of the Abstract Book. Thank you to Stephanie Cleven and Robbin Grouwels from Klinkhamer Group for the smooth collaboration.

Only two years after WC12, we are excited to be meeting for the first time in South America in the energetic, cosmopolitan city of Rio de Janeiro, also known as the *Cidade Maravilhosa* (Marvellous City in Portuguese). We wish all participants of WC13 a captivating congress that stimulates new ideas and novel collaborations in our joint effort to replace, reduce, and refine animal experiments towards human-relevant and humane science.

We can now already start looking forward to WC14, which will bring us together in Seoul, South Korea in 2027.

With best wishes,

Sonja von Aulock
Editor in chief, ALTEX & ALTEX Proceedings



Octavio A. F. Presgrave

Chantra Eskes

Marize Valadares

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Alternatives Congress Trust

Welcome

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Oral Presentation numbers per theme and session

Animal Health

Monday 1 Sept., 09.30-11.00, room 205.

Session 118. Ethics in Action: Upholding the 3Rs through Animal Research Ethical Review.

Chairs: Jessica Cait, Aileen MacLellan.

Oral presentation abstract numbers: **177, 231, 336, 143.**

Monday 1 Sept., 11.30-13.00, room 205.

Session 56. Working together to end 'severe' suffering.

Chair: Karynn Capilé.

Oral presentation abstract numbers: **578, 571, 43, 429.**

Monday 1 Sept., 14.30-16.00, room 205.

Session 94. Fishing for the 3Rs? Why using fish should not count as refinement and their suffering does matter.

Chairs: Barbara Jozef, Jessica Rosolowski.

Oral presentation abstract numbers: **535, 226, 569, 718, 270.**

Tuesday 2 Sept., 09.30-11.00, room 205.

Session 26. One world, many standards: international perspectives on regulating the use of animals in science.

Chairs: Barney Ree, Marcel Frajblat.

Oral presentation abstract numbers: **748, 432, 261, 749, 139.**

Tuesday 2 Sept., 11.30-13.00, room 205.

Session 99. Phase out of animal testing in veterinary vaccines batch release test – An overview of Multi-stakeholders efforts.

Chair: Laura Viviani.

Oral presentation abstract numbers: **392, 130, 517, 428, 617.**

Tuesday 2 Sept., 14.30-16.00, room 205.

Session 55. 3Rs Implementation in Brazil: a Successful Multi-stakeholder Joint Effort.

Chairs: Luisa Macedo Braga, Bianca Marigliani.

Oral presentation abstract numbers: **652, 544, 519, 480.**

Wednesday 3 Sept., 09.30-11.00, room 205.

Session 60. How systematic reviews and meta-analyses support good animal welfare and the transition to animal-free research.

Chair: Ernesto Gulin.

Oral presentation abstract numbers: **276, 97, 151, 388.**

Wednesday 3 Sept., 11.30-13.00, room 205.

Session 74. Bias, Reporting and Assessment – How to replace, reduce and refine animal experiments by looking at the available data.

Chairs: Tilo Weber, Laura Alvarez.

Oral presentation abstract numbers: **441, 560, 253, 400, 595.**

Education

Monday 1 Sept., 09.30-11.00, room 210.

Session 71. Integrating all 3Rs at Research Institutions through Training, Education, and Culture.

Chairs: Adrian Smith, Megan LaFollette.

Oral presentation abstract numbers: **33, 152, 141, 132, 158.**



Monday 1 Sept., 11.30-13.00, room 210.

Session 32. "Eye-opening" education for experimenters.

Chair: Derek Fry.

Oral presentation abstract numbers: **316, 185, 416.**

Monday 1 Sept., 13.00-14.00, room 210.

Session 110. Lunch Session – Workshop to expand the educational and geographical coverage of the Global Education Hub for animal-free innovation.

Chairs: Daniela Salvatori, Cyrille Krul.

Oral presentation abstract number: **424.**

Monday 1 Sept., 14.30-16.00, room 210.

Session 122. Building an Ethical and Innovative Career: Guidance for Biology, Veterinary, and Medical Students and Professionals.

Chairs: Kathrin Herrmann, Nick Jukes.

Oral presentation abstract numbers: **19, 638, 407, 425.**

Tuesday 2 Sept., 09.30-11.00, room 207.

Session 39. Alternative Models for Surgical Training.

Chair: Marcelo Asprea.

Oral presentation abstract numbers: **532, 621, 557.**

Tuesday 2 Sept., 11.30-13.00, room 207.

Session 43. Hands on Training in NAMs to Enhance Translational Success.

Chairs: Mohammad Akbarsha, Lena Smirnova.

Oral presentation abstract numbers: **285, 355, 678, 449, 242.**

Tuesday 2 Sept., 13.00-14.00, room 207.

Session 119. Lunch Session – Next Generation Risk Assessment for Fragrance Materials.

Chair: Anne Marie Api.

Oral presentation abstract numbers: **332, 686, 348, 333.**

Tuesday 2 Sept., 14.30-16.00, room 207.

Session 54. Empowering the Next Generation of Researchers for Next Generation Risk Assessment.

Chairs: Mathieu Vinken, Eliska Kuchovska.

Oral presentation abstract numbers: **51, 347, 39, 79, 172.**

Wednesday 3 Sept., 09.30-11.00, room 203.

Session 174. 3Rs around the world.

Chairs: Jeffrey Bajramovic, Winfried Neuhaus.

Oral presentation abstract numbers: **529, 382, 145, 666.**

Wednesday 3 Sept., 14.30-16.00, room 203.

Session 81. Breaking Down Silos to Achieve One Health – Integrating Environment into Education and Training Initiatives in Latin America and Beyond.

Chairs: Renato Ivan de Ávila, Luciene Bottentuit Lopez Balottin.

Oral presentation abstract numbers: **258, 463, 515, 541, 492.**

Thursday 4 Sept., 09.30-11.00, room 203.

Session 144. Empowering Change through Informal Education: Leveraging Workshops and Webinars to Promote the 3Rs.

Chairs: Camila Sgrignoli Januario, Kathrin Herrmann.

Oral presentation abstract numbers: **248, 282, 284, 342, 562.**



Environmental Health

Monday 1 Sept., 09.30-11.00, room 207.

Session 61. Opportunities for Data-Driven Enhancement Across Human and Environmental Safety Assessment.

Chairs: Marissa Kosnik.

Oral presentation abstract numbers: **34, 89, 110, 247, 468.**

Monday 1 Sept., 11.30-13.00, room 207.

Session 42. Complex chemical exposure – How can in vitro bioassays improve current practice in human biomonitoring and environmental monitoring?

Chairs: Beate Escher, Enrico Mendes Saggiaro.

Oral presentation abstract numbers: **674, 67, 73, 602, 491, 635.**

Monday 1 Sept., 14.30-16.00, room 207.

Session 63. Marine Environmental Safety: recent advances in NAMs to evaluate toxicity of chemicals of emerging concern in marine environments.

Chairs: Gisela Umbuzeiro, Dâmaris Santos.

Oral presentation abstract numbers: **40, 75, 95, 422.**

Wednesday 3 Sept., 11.30-13.00, room 207.

Session 40. Environmental safety assessment of chemicals – Progress of different animal-free tools, similarities and differences with human safety assessments.

Chairs: Veronique Poulsen, Donna Macmillan.

Oral presentation abstract numbers: **281, 398, 752, 221.**

Wednesday 3 Sept., 14.30-16.00, room 207.

Session 138. Moving forward towards the application of New Approach methodologies for the environmental risk assessment of chemicals.

Chairs: Lena Smirnova, Helena Hogberg, Vanessa Rocha.

Oral presentation abstract numbers: **548, 549, 55, 447, 453.**

Thursday 4 Sept., 9.30-11.00, room 207.

Session 107. Accelerating Science to Policy Transfer of Animal Alternatives for human and Environmental safety assessment: lessons learned and ways forward.

Chairs: Bruno Campos, Jose Tarazona.

Oral presentation abstract numbers: **112, 414, 746, 601.**

Human Health – 3Rs

Tuesday 2 Sept., 09.30-11.00, room 210.

Session 33. Generative AI for Drug Safety and Toxicology.

Chairs: Nicole Kleinstreuer, Lena Smirnova.

Oral presentation abstract numbers: **7, 87, 623, 690.**

Tuesday 2 Sept., 11.30-13.00, room 210.

Session 134. Exposure-based human risk assessment to minimize unnecessary animal testing for agrochemicals.

Chairs: Shadia M. I. Catalano, Sandrine Deglin.

Oral presentation abstract numbers: **356, 255, 688, 759, 254.**

Tuesday 2 Sept., 14.30-16.00, room 210.

Session 92. Integrated strategy for DART New Approach Methods.

Chair: Susana Proença.

Oral presentation abstract numbers: **587, 41, 537, 308.**



Wednesday 3 Sept., 09.30-11.00, room 210.

Session 62. The Continuum of New Approach Methodologies from research to regulation.

Chair: Nicole Kleinstreuer.

Oral presentation abstract numbers: **287, 586, 413, 420.**

Wednesday 3 Sept., 11.30-13.00, room 210.

Session 5. The role of Sentience in shaping policy in animal research.

Chairs: Paul Locke, Rebecca Critser.

Oral presentation abstract numbers: **584, 563, 334.**

Wednesday 3 Sept., 11.30-13.00, room 203.

Session 87. European Partnership on Alternative Approaches to Animal Testing (EPAA): 20 years of progress.

Chairs: Gavin Maxwell, Christian Desaintes.

Oral presentation abstract numbers: **591, 421, 369, 540, 313.**

Wednesday 3 Sept., 14.30-16.00, room 210.

Session 53. Challenges in Asia to adopting the 3Rs across areas of animal use.

Chairs: Yasuyuki Sakai, Seokjoo Yoon.

Oral presentation abstract numbers: **201, 202, 301, 452.**

Thursday 4 Sept., 09.30-11.00, room 210.

Session 117. The 5Rs of Developmental Immunotoxicity Testing: Advancing Ethical and Predictive Methods.

Chairs: Fenna Sillé, Vic Johnson.

Oral presentation abstract numbers: **186, 207, 25, 594, 647.**

Human Health – Policies

Monday 1 Sept., 09.30-11.00, room 201.

Session 84. Transitioning to Animal-Free Chemical Safety Assessment.

Chairs: Gavin Maxwell, Kristie Sullivan.

Oral presentation abstract numbers: **411, 192, 493, 573, 503, 105.**

Monday 1 Sept., 11.30-13.00, room 201.

Session 125. Ending 100 years of suffering: Regulatory assessment of Acute Oral Toxicity without using animals.

Chairs: Jay Ingram, Kamel Mansouri.

Oral presentation abstract numbers: **101, 629, 460, 551, 498.**

Monday 1 Sept., 14.30-16.00, room 201.

Session 127. Regulatory Frameworks for Complex Endpoints.

Chairs: Helena Hogberg, Donna Macmillan.

Oral presentation abstract numbers: **113, 607, 239, 643.**

Tuesday 2 Sept., 09.30-11.00, room 201.

Session 75. Update to OECD Guidance Document 34 on the validation of test methods and Defined Approaches.

Chairs: Alison Harrill, João Barroso.

Oral presentation abstract numbers: **277, 22, 154, 178.**

Tuesday 2 Sept., 11.30-13.00, room 201.

Session 48. Confidence in *in vitro* alternatives to animal testing – defining standards to ensure rigorous and reproducible data.

Chairs: Susanne Kolle, Amanda Ulrey.

Oral presentation abstract numbers: **349, 338, 353, 361, 306, 577.**

Tuesday 2 Sept., 14.30-16.00, room 201.

Session 143. Sailing the Seas of Validation: Challenges Encountered to Chart the Course Ahead.

Chairs: David Allen, Nathalie Alépée.

Oral presentation abstract numbers: **518, 435, 224, 155, 244.**



Wednesday 3 Sept., 09.30-11.00, room 201.

Session 106. The bigger picture of NAM uptake: exploring psychological and system factors as promoters and inhibitors.

Chairs: Sebastian Hoffmann, Gro Haarklou Mathisen.

Oral presentation abstract numbers: **507, 233, 714, 479.**

Wednesday 3 Sept., 09.30-11.00, room 202.

Session 113. Overcoming barriers to regulatory uptake of Next Generation Risk Assessment.

Chairs: Catherine Willet, Gladys Ouedraogo.

Oral presentation abstract numbers: **328, 327, 488, 271, 747.**

Wednesday 3 Sept., 11.30-13.00, room 201.

Session 29. Building Confidence and Transparency into Validation Management and Peer Review.

Chairs: Emily Reinke, Amanda Ulrey.

Oral presentation abstract numbers: **31, 157, 82, 91.**

Wednesday 3 Sept., 11.30-13.00, room 202.

Session 109. From internal decision making to regulatory approval: Finding strategies to accelerate the use of NAMs.

Chairs: Kristie Sullivan, Nicolas Fabre.

Oral presentation abstract numbers: **324, 502, 289, 299, 246.**

Wednesday 3 Sept., 14.30-16.00, room 201.

Session 15. Regulatory experience using a weight of evidence approach to reducing testing on animals.

Chairs: Gina Hilton, Marco Corvaro.

Oral presentation abstract numbers: **368, 103, 510, 568.**

Wednesday 3 Sept., 14.30-16.00, room 202.

Session 98. Transitioning away from animal testing for vaccine quality control testing: international and regional perspectives.

Chairs: Laura Viviani, Elliot Lilley.

Oral presentation abstract numbers: **68, 393, 626, 245, 197.**

Thursday 4 Sept., 09.30-11.00, room 201.

Session 133. Innovations in Microphysiological Systems (MPS): Advancing Translational Research and Regulatory Risk Assessment.

Chairs: Elizabeth Baker, Charu Chandrasekera.

Oral presentation abstract numbers: **16, 37, 262, 476, 310, 60.**

Thursday 4 Sept., 09.30-11.00, room 202.

Session 67. Regulatory Acceptance of Alternatives to Animal Testing.

Chairs: Elkiene Macedo Rama, Steven Musser.

Oral presentation abstract numbers: **757, 758, 198, 83, 23.**

Human Health – Replacement

Monday 1 Sept., 09.30-11.00, plenary room.

Session 20. Artificial Intelligence to reduce and replace animal testing.

Chair: Karen Cazarin.

Oral presentation abstract numbers: **187, 405, 180, 196.**

Monday 1 Sept., 09.30-11.00, room 202.

Session 64. Accelerating regulatory acceptance of complex manufactured and natural products through New Approach Methodologies (NAMs).

Chairs: Philip Marx-Stoelting, Daniela Morais Leme.

Oral presentation abstract numbers: **146, 142, 691, 80.**



Monday 1 Sept., 11.30-13.00, plenary room.

Session 135. Next-Generation Risk Assessment (NGRA).

Chair: Leonardo Pinto.

Oral presentation abstract numbers: **396, 676, 181, 471.**

Monday 1 Sept., 11.30-13.00, room 202.

Session 77. Development of non-animal methods for the identification of respiratory sensitizers: Reconciling the differences in clinical diagnosis and regulatory policies.

Chairs: Kristie Sullivan, Nikaeta Sadekar.

Oral presentation abstract numbers: **430, 296, 675, 478.**

Monday 1 Sept., 13.00-14.00, room 202.

Session 142. Lunch Session – Accelerating rare disease therapeutics via adoption of human biology-based non-clinical drug development in global north and global south countries.

Chair: Janette Turner.

Oral presentation abstract numbers: **553, 323, 459, 464.**

Monday 1 Sept., 14.30-16.00, plenary room.

Session 103. ASPA, the guided workflow for next-generation chemical safety assessment.

Chairs: Bob van de Water, Giorgia Pallocca.

Oral presentation abstract numbers: **581, 680, 489, 712, 687.**

Monday 1 Sept., 14.30-16.00, room 202.

Session 25. 3D Bioprinting Put Towards Tissue Model Fabrication.

Chairs: Mario Alvarez, Y. Shrike Zhang.

Oral presentation abstract numbers: **681, 20, 750, 458, 9.**

Tuesday 2 Sept., 09.30-11.00, plenary room.

Session 79. Using New Approach Methods (NAMs) to derive point-of-departures (PoD) for Next Generation Risk Assessment (NGRA) of skin sensitizers: towards regulatory implementation.

Chairs: Andy Forreryd, Renato Ivan de Ávila.

Oral presentation abstract numbers: **120, 138, 93, 92.**

Tuesday 2 Sept., 09.30-11.00, room 202.

Session 44. Advanced Strategies for Implementation of the 3R Principles in Genotoxicity Assessment.

Chairs: Paul White, Stefan Pfuhler.

Oral presentation abstract numbers: **582, 472, 512, 604, 504.**

Tuesday 2 Sept., 11.30-13.00, plenary room.

Session 13. The journey towards animal-free systemic toxicity assessment for cosmetics.

Chairs: David Allen, Gladys Ouedraogo.

Oral presentation abstract numbers: **1, 58, 69, 104, 149.**

Tuesday 2 Sept., 11.30-13.00, room 202.

Session 24. New Approach Methodologies (NAMs) for Assessing Reproductive and Developmental Toxicity.

Chair: Kelen Fabiola Arrotei.

Oral presentation abstract numbers: **592, 677, 12, 418, 513.**

Tuesday 2 Sept., 13.00-14.00, room 202.

Session 6. Lunch Session – Safety Assessment of Natural Complex Substances using New Approach Methodologies (NAMs).

Chair: Nathalia de Carvalho Indolfo.

Oral presentation abstract numbers: **685, 679.**



Tuesday 2 Sept., 13.00-14.00, room 210.

Session 93. Lunch Session – Developing disease models with local relevance: 3R model development in Africa.

Chairs: Stefanie Klima, Adele Nel.

Oral presentation abstract numbers: **222, 108, 136.**

Tuesday 2 Sept., 14.30-16.00, room 202.

Session 46. Reflecting Human Diversity with In Vitro Models.

Chairs: Maren Schenke, Lena Smirnova.

Oral presentation abstract numbers: **6, 59, 598, 278, 366.**

Wednesday 3 Sept., 09.30-11.00, plenary room.

Session 102. The Future is Now: The Power of NAMs for a global acceptance for Local Tolerance.

Chair: Vanja Dakic.

Oral presentation abstract numbers: **273, 46, 267, 290.**

Wednesday 3 Sept., 11.30-13.00, plenary room.

Session 101. Formulating a framework for non-animal safety assessment of human pharmaceuticals.

Chair: Jan Turner.

Oral presentation abstract numbers: **275, 326, 118, 683.**

Wednesday 3 Sept., 13.00-14.00, room 210.

Session 23. Lunch Session – Recent advances on bioprinting skin tissue models for toxicology and drug evaluation.

Chairs: Daniel Nieto, Nadina Usseglio.

Oral presentation abstract numbers: **204, 126, 234, 456.**

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Session 22. Implementation of NAM based PBK modelling into regulatory hazard assessment.

Chair: Sylvia Escher.

Oral presentation abstract numbers: **140, 173, 753, 291, 250.**

Wednesday 3 Sept., 14.30-16.00, room 205.

Session 100. Animal Replacement Technologies Down Under – Unifying Science, Innovation, and Humanity for a Sustainable Future.

Chair: Wojciech Chrzanowski.

Oral presentation abstract numbers: **133, 77, 443, 206, 444, 455.**

Thursday 4 Sept., 09.30-11.00, plenary room.

Session 69. Replacing invisible animal suffering – Animal-free materials in cell and tissue culture.

Chairs: Tilo Weber, Jeffrey Bajramovic.

Oral presentation abstract numbers: **377, 28, 27, 184, 409.**

Thursday 4 Sept., 09.30-11.00, room 205.

Session 17. Session on Current Research Initiatives on NAMs in Korea and Global Cooperation for Regulatory Application.

Chairs: Kyung-Chul Choi, Jung Min Choi.

Oral presentation abstract numbers: **135, 134, 129, 45, 159.**

One Health

Monday 1 Sept., 09.30-11.00, room 203.

Session 30. Global Harmonization in Risk Assessment: Navigating the Complexities of Animal Testing, Regulatory Divergence, and Environmental Sustainability.

Chairs: Arianna Giusti, Ariadne Moraes.

Oral presentation abstract numbers: **567, 312, 114, 176.**



Monday 1 Sept., 11.30-13.00, room 203.

Session 132. Application of New Approach Methods in the Safe and Sustainable Design of Innovative Products.

Chairs: Barry Hardy, Carolina Horta Andrade.

Oral presentation abstract numbers: **179, 280, 477, 531, 713.**

Monday 1 Sept., 14.30-16.00, room 203.

Session 126. One Health, Many Models: Computational Approaches for Systemic Toxicities.

Chair: Antonio Anax Falcão de Oliveira.

Oral presentation abstract numbers: **495, 2, 213, 102.**

Tuesday 2 Sept., 09.30-11.00, room 203.

Session 41. NAMs as tools to operationalize One Health in human toxicology and ecotoxicology.

Chair: Jinhee Choi.

Oral presentation abstract numbers: **341, 205, 543, 194.**

Tuesday 2 Sept., 11.30-13.00, room 203.

Session 78. Unveiling Chemical Similarity in Computational Toxicology: Navigating Supervised and Unsupervised Approaches.

Chairs: Kamel Mansouri, José L. Medina-Franco.

Oral presentation abstract numbers: **100, 74, 501, 533.**

Tuesday 2 Sept., 14.30-16.00, room 203.

Session 9. Current and Future Directions for Human and Environmental Exposure Assessment in Next Generation Risk Assessment: From External to Internal Approaches Across Regulatory Silos.

Chairs: Abdulkarim Najjar, Arianna Giusti.

Oral presentation abstract numbers: **309, 465, 243, 373.**

Wednesday 3 Sept., 09.30-11.00, room 207.

Session 36. Leveraging inter-disciplinary and human-relevant approaches to address mental health.

Chairs: Sandra Coecke, Francesca Pistollato.

Oral presentation abstract numbers: **339, 305, 346, 538, 5.**

Wednesday 3 Sept., 13.00-14.00, room 202.

Session 4. Lunch Session – 3Rs in space: animal-free alternatives for life sciences in space research.

Chairs: Mathieu Vinken, Danilo Tagle.

Oral presentation abstract numbers: **4, 3, 21.**

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Oral Presentations

1

Assessing analogue suitability and mode of action through integration of diverse types of data: Implications for next generation risk assessment

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In our first case study we assessed the bioactivity and reactivity of benzoic acid analogues and their suitability for read-across assessments. Matched Molecular Pair (MMP) and Quantitative Similarity (qSim) approaches were used to categorize benzyl derivatives based on their chemical structures. Metabolism data played a crucial role in defining the category. The study found that benign salts of benzoic acid, para- and meta-toluic acids, displayed the same metabolism as benzoic acid and had the highest TotalScore of 3.0. Small alkyl derivatives of benzoic acid showed small differences in metabolism and had a TotalScore ranging from < 3.0 to ≥ 2.5 . The study also utilized Pharmacology Profiling, ToxCast data, and Transcriptomic data to substantiate the low bioactivity of benzoic acid analogues and demonstrate differences in reactivity for analogues outside the read-across category.

In a second case study we focused on exploring the biological activity of avobenzone (AVB), a widely used sunscreen in cosmetic products. The objective was to understand the mode of action (MoA) of AVB by investigating its functional analogs. Suitable structural analogs for AVB were not identified, so ToxCast data and literature were used to identify potential functional analogs. The transcriptional profile of AVB and related compounds was determined in various cell types, and connectivity map (cMAP) analysis was performed. *In vitro* pharmacology profiling was also conducted to evaluate AVB's potential interaction with defined targets. The study revealed that AVB targeted mitochondrial depolarization, supported by the activation of TOX21_MMP_ratio. Functional analogs, such as 2-, 3-, and 4-Hydroxybenzophenone (HDBP) and flavonoids kaempferol (KA) and myricetin (MY), showed similar effects. cMAP analysis indicated that AVB and MY elicited a transcriptional profile similar to mitochondrial uncouplers, while HDBP and KA exhibited similarities to estrogen and/or androgen receptor agonists and antagonists. AVB showed no significant gene expression changes at lower concentrations. The study concluded that the primary MoA of AVB is mitochondrial perturbation leading to cytotoxicity, and *in vitro* test concentrations of 5-10 μM were considered to represent no biological activity for AVB.

Collectively these case study findings emphasize the opportunities for improving the accuracy of analogue identification and integrating NAM to enhance traditional risk assessments.

Presentation: Oral

2

A One Health mindset to transform agrochemical safety evaluation through application of modern approaches to testing and assessment

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The present safety evaluation paradigm for agrochemicals is long-standing and anchored in well-established testing and evaluation procedures. A transformation from the current frameworks is needed that applies modern evaluation strategies to better support sustainable agriculture. This will be accomplished through the integration of state-of-the-art scientific methods, technologies and data sources, to inform safety and risk decisions for robust human and environmental safety and risk assessment of agrochemicals. The success of this change requires reframing the safety evaluation into one that incorporates new scientific tools and methodologies, interpreted through a One Health lens, that ensures the safety of crop protection products for both humans and the environment.

Presentation: Oral



3

New approach methodologies used for ground-based life sciences in space research

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Despite more than 6 decades of human spaceflight, several aspects related to the effects of space conditions, in particular low gravity and radiation, on human physiology have still not been fully elucidated. Although not explicitly named as such, new approach methodologies (NAMs) have been used for many years in life sciences in space research. However, as the result of progressive interdisciplinary collaboration and a rapid succession of technological innovations, new NAMs have been introduced at high pace over the past decade. At present, NAMs are mainly used in life sciences in space research to model human biological and physiological responses to space conditions in both real-life and simulated environments. These range from simple monolayer cultures of individual primary or stem cells all up to bioprinted 3D organoids and microfluidic chips that recapitulate the complex cellular architecture of organs. Other approaches used in parallel do not fully replace animal testing *per se* but rather contribute to the reduction of animal experimentation. These include tools to mimic space conditions on Earth, such as microgravity and radiation simulators, as well as tools to support the processing, analysis or application of testing results obtained in life sciences in space research, including systems biology, live-cell, high-content and real-time analysis, high-throughput analysis, artificial intelligence and digital twins. An overview of these NAMs and tools, *in casu* used for ground-based life sciences in space research, will be provided in the present presentation.

Presentation: Oral

4

Animal testing in life sciences in space research: A historical and critical overview

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Animals have been flown to space since the 1940s and have become a part of the space race. Experimentation provided data pivotal for the flights of the first astronauts and cosmonauts, with studies conducted onboard biosatellites, space shuttles, and the International Space Station. The commercialization and acceleration of spaceflight frequency have enabled a “second space age”. Research has shifted to multi-omics, single-cell, and precision medicine studies. The talk will present the history of animal spaceflight, along with their role in reducing the risk of human space exploration in low Earth orbit and beyond. It will also describe how studies of animals in space enabled research and discovery for the benefit of humans on Earth, an application that was not anticipated during the early days of animal experimentation in space. Finally, it will provide an outlook for the future of studies on animals in space. The focus will be on past and future ethical considerations.

Presentation: Oral



5

Integrative approaches to mental health: The role of healthcare, social inclusion, education, and culturally sensitive initiatives

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In recent years, mental health issues, particularly depression, have emerged as a significant public health concern affecting individuals across all demographics. The World Health Organization has recognized mental health as a critical component of overall well-being, yet stigma, lack of access to care, and social determinants continue to hinder effective treatment. This presentation explores how an interdisciplinary approach – combining healthcare, social inclusion, education, and culturally sensitive initiatives – can collectively address mental health challenges. By fostering collaboration across sectors, we can create a more supportive environment that promotes mental well-being and resilience.

The intersection of healthcare and mental health is paramount in addressing the rising rates of depression. Integrated care models, which combine mental health services with primary healthcare, have shown promising results in improving access and outcomes for individuals in need. By training primary care providers to recognize and address mental health issues, we can ensure that patients receive holistic care that considers both their physical and mental health needs. This presentation will delve into successful case studies where integrated care has led to improved patient outcomes, emphasizing the importance of early intervention and continuous support.

Education plays a vital role in shaping attitudes and understanding around mental health. Mental health literacy programs in schools can equip students, educators, and parents with the knowledge to recognize signs of mental distress and seek help. By incorporating mental health education into the curriculum, we can reduce stigma and foster a culture of openness and support. This workshop will discuss effective strategies for implementing mental health literacy initiatives in educational settings, showcasing examples of schools that have successfully integrated these programs and the positive impact on student well-being.

Social inclusion is another critical component in addressing mental health issues. Marginalized communities often face barriers to accessing mental health care, including economic instability, discrimination, and lack of social support. Community engagement initiatives that promote social inclusion can help bridge these gaps. This presentation will explore various community-driven mental health programs that have successfully engaged diverse populations, highlighting the role of peer support networks and community organizations in providing accessible and culturally relevant mental health resources. By fostering strong community ties, we can create environments that support mental health and well-being for all individuals.

Culturally sensitive practices are essential in ensuring that mental health services are effective and equitable. Mental health professionals must be trained to understand and respect the diverse cultural backgrounds of their clients. This includes recognizing how cultural beliefs and practices influence perceptions of mental health and treatment. This presentation will emphasize the importance of culturally responsive care and provide insights into training programs that prepare mental health providers to deliver care that is respectful of and tailored to the needs of diverse populations.

Presentation: Oral

6

***In vitro* models for human genetic diversity: Case examples of utility for decision-making**

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Es early as 1960s, the regulatory bodies recognized that “genetically determined differences in the enzymatic pattern of man can affect his reaction to toxic agents;” thus, some form of accounting for population variability in quantitative risk assessment was needed. Both US and international agencies adopted an empirical method to address such variability using default “factors”, generally 10. While the default factors are uniformly disliked, they continue to be used in risk assessment of both drugs and chemicals. Sequencing of the human genome revolutionized the field of biomedical sciences. Thousands of studies have focused on identifying susceptibility loci and identifying those who are at risk of adverse drug reactions. Personalized medicine is relying heavily on modern sequencing methods to characterize the genetic makeup of the host and/or disease. However, less progress has been achieved in discovering actionable gene-environment interactions that can inform decision-making in chemical risk assessment. Development of experimental model systems and organisms that can experimentally quantify population variability is one area of measurable progress to move away from defaults. Progress is most evident in the use of population *in vitro* human cell-based models. Multiple case studies were published in the past decade that demonstrate the utility of such models for quantifying variability at hazard identification, dose-response assessment, and mechanistic understanding steps of risk assessment. This presentation will review recent efforts to develop fit-for-purpose population-based *in vitro* models in the context of regulatory science. It will also describe key challenges and opportunities for broadening application of population-based experimental approaches to additional organ and cell types.

Presentation: Oral



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Development of virtual animal models with generative AI for drug safety and toxicology

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Testing data from laboratory animals provide crucial evidence for safety evaluation of therapeutics and risk assessment of environmental chemicals. This animal testing paradigm has been an essential component in regulating drug, food, and chemical safety by almost all the regulatory agencies in the world. As a result, abundant animal data are available from public domain and private practice. As the toxicology community and regulatory agencies are moving towards reduction, refinement and replacement (3Rs principle) of animal studies, we are exploring Generative Artificial Intelligence (GenAI) approaches to learn from existing animal studies so that it can generate the animal data as virtual animal models. This presentation will showcase examples from FDA projects using GenAI, focusing on its application in drug safety. Specifically, these projects apply GenAI to (1) create virtual animals as digital twins in toxicology, (2) generate toxicogenomics data based solely on experimental design; and (3) conduct translational toxicology research. We will discuss how these GenAI methods to advance the 3Rs in toxicology, drug safety assessment, and regulatory science.

Presentation: Oral

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3D bioprinting for engineering high-content human-based tissue models

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Over the last decades, the fabrication of three-dimensional (3D) tissues has become commonplace. However, conventional 3D fabrication techniques are limited in their capacity to produce complex tissue constructs with the required precision and controllability that is needed to replicate biologically relevant tissues. To this end, 3D bioprinting offers great versatility in the fabrication of biomimetic volumetric tissues that are structurally and functionally relevant. It enables precise control of the composition, spatial distribution, and architecture of bioprinted constructs facilitating the recapitulation of the delicate shapes and structures of target organs and tissues. This talk will discuss our recent efforts on developing various bioprinting methods that allow high-content biofabrication of microphysiological systems *in vitro* to recapitulate the important biological and physiological parameters of their *in vivo* counterparts. These biomimetic microtissues are anticipated to supplement the conventional planar, static cell cultures, and to bridge the gaps between the current pre-clinical animal models and the human body, likely providing new opportunities in constructing functional tissue and disease models for drug discovery, therapeutics screening, and precision medicine.

Presentation: Oral



12

NAMs using microphysiological systems as drug development tools for reproductive health

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Introduction: Clinical and technological advancements have not reduced the rate of adverse pregnancy outcomes like preterm birth (PTB; birth before 37 weeks), which affects 15 million pregnancies globally and contributes to 1 million neonatal deaths annually. Limitations of the current *in vitro* models to recreate fetomaternal interface (FMI) as seen during pregnancy and our dependency on animal models that do not recapitulate human pregnancy have resulted in ongoing crises limiting us from providing proper interventions during pregnancy to reduce PTB risk. New Approach Methods (NAMs) are needed to reduce, refine, and replace animal models and provide reliable and reproducible data to mitigate PTB risk. For this, we developed a microphysiological system (MPS) based multi-organ fetal membrane-placenta organ-on-chip (FMI-PLA-OOC) to establish physiologic and pathologic models of the FMI.

Methods and results: FMI-PLA-OOC contained cells from human umbilical cord endothelium, cyto- and syncytiotrophoblasts, decidua, chorion, and amnion (mesenchyme and epithelium) in seven cell chambers connected by arrays of collagen-filled microchannels. As infection and inflammation are risk factors for PTB, we determined the differential responses of fetal and maternal cells of the FMI to an infectious stimulus in producing an inflammatory response. Lipopolysaccharide (LPS; 100 ng/mL) was used to mimic infection, propagation was documented by immunostaining, and cytokine assays measured inflammation. Additionally, to test the rigor of our model and its utility for preclinical trials, we also tested exosomal encoded anti-inflammatory cytokine interleukin-10 (eIL-10) to reverse the adverse effects of infection-induced inflammation at the FMI. We first validated FMI-PLA-OOC by determining cytotoxicity (lactate dehydrogenase [LDH] of cells and viability on the chip and tested for in utero cellular characteristics (immunocytochemistry [ICC]). Our data suggest that markers and viability of cells in the FMI-PLA-OOC were like in-utero FMI, validating the device's physiological relevance. LPS propagated from maternal to fetal cells within 6 days, inducing a progressive increase in pro-inflammatory cytokine concentrations (IL-6, IL-8, TNF- α) and a decrease in anti-inflammatory cytokine production (IL-10) across the fetal membrane and placenta layers. eIL-10 propagated from the maternal to the fetal side, and this treatment reduced inflammation in both the fetal and maternal compartments. eIL-10 did not cause any cytotoxicity or reduce cellular viability.

Conclusions: Our MPS model established healthy and disease models of FMI using a single *in-vitro* platform. Further, we showed the utility of this platform for preclinical trials to mitigate inflammation induced by infection. This platform provides broad utility in obstetrics research as a drug development tool in reproductive health.

Presentation: Oral

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Oxygen saturation measurement in blood-filled vascular structures embedded in a multi-organ-chip

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Animal experiments and traditional cell culture methods face significant challenges in replicating human systemic interactions. Multi-Organ-Chip systems overcome these limitations by providing human-relevant, systemic models for biomedical research.

This work presents a novel methodology to fabricate vascularized structures directly within a compartment of a Multi-Organ-Chip. Using a custom-designed microscope, photolithographic techniques were developed to print vascular channels with precise control over geometry, including a novel height-limitation approach to achieve accurate structural dimensions. These channels were further optimized to support fluid flow, physiological wall shear stress, and endothelial cell seeding.

In addition, a reflection pulse oximetry-inspired method was implemented to measure oxygen saturation within the blood-filled vascular channels. This approach enabled real-time analysis of oxygen transport and aerobic metabolism in the hydrogel-embedded vascular network.

The results demonstrate the successful fabrication of functional vascular structures within the hydrogel, capable of maintaining dynamic flow conditions and facilitating oxygen exchange. Measured oxygen saturation gradients confirmed effective oxygen transport and metabolic activity, showcasing the system's physiological relevance.

This innovative methodology lays the groundwork for developing complex, vascularized organ models within Multi-Organ-Chips, while providing a platform to monitor their oxygen dynamics in real-time. Such advancements represent a critical step toward reducing animal experiments and enabling more accurate human tissue modeling for drug discovery and personalized medicine.

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Presentation: Oral



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Beyond the lab: Driving change through ethical science and animal-free research

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Building an ethical and innovative career in the life sciences requires resilience, creativity, and a willingness to challenge the status quo. Drawing from my own journey – spanning scientific research, advocacy, and multifaceted roles at Doctors Against Animal Experiments (DAAE) – I will discuss the realities and rewards of pursuing non-traditional career paths. I will share personal experiences with the challenges of publishing on controversial topics and the unique opportunities that arise at the intersection of NGOs and science, where campaigners can drive meaningful change. Using drug attrition as a case study, I will illustrate how high failure rates in clinical trials persist in the last six decades and that the major reasons for drug failure in humans are parameters deemed favorable in animal-based preclinical testing, highlighting the urgent need for more human-relevant methods. I will introduce key resources and databases that support students and professionals in identifying animal-free research methods and innovative companies. Additionally, I will showcase successful campaigns revealing strong patient opposition to animal testing, and present our Eastern Europe project, which leverages computational approaches to reduce animal use. The global shift toward animal-free science – reflected in policy changes and funding priorities in the USA and EU – demonstrates a growing recognition of both ethical imperatives and scientific limitations of animal models. Finally, I will highlight how diverse backgrounds and fields can contribute to ethical progress in science, encouraging students and professionals to embrace interdisciplinary, impactful careers that align with their values.

Presentation: Oral

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Nanofiber-integrated 3D printing for *in vitro* tissue modeling

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Generation of *in vitro* volumetric tissue models is challenging. Here, we present a method for expanding microchannel-embedded paper devices using a precisely controlled gas-foaming technique to create volumetric tissue models *in vitro*. Hollow, perfusable microchannel patterns were successfully fabricated within a densely entangled network of bacterial cellulose nanofibrils through matrix-assisted sacrificial 3D printing. These microchannel patterns maintained their structural integrity following expansion in an aqueous NaBH₄ solution enabled by gas foaming. The expanded devices exhibited multilayered laminar structures with thicknesses that could be adjusted based on NaBH₄ concentration and expansion duration. Expansion significantly increased both the thickness and porosity of the bacterial cellulose network, facilitating enhanced cellular infiltration compared to non-expanded devices. This straightforward approach offers a cost-effective means of generating volumetric, human-relevant tissue models, such as vascularized tumor constructs, with potential applications in preclinical drug testing and personalized therapy development.

Presentation: Oral



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New approach methodologies used at the International Space Station

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A number of human physiological changes caused by microgravity include bone demineralization, skeletal muscle atrophy, vestibular problems, cardiovascular issues that can result in postflight orthostatic intolerance, immunosenescence, and reductions in plasma volume and red cell mass. Space exploration is a new frontier with long-term missions to the moon and Mars not far away. Understanding and modeling the physiological changes caused by long-duration microgravity remains a daunting challenge. The use of animals, including non-human primates, have been utilized in the past when studying the physiological responses involved in the adaptation to changes in gravitational loading. Understanding the fundamental mechanisms of adaptation to microgravity-related effects at tissue, cellular, and molecular levels is one of the most essential scientific problems for space medicine. Resemblance of the physiological changes occurring during space flight to the hallmarks of human aging is of special interest that raises a crucial question about the similarity of underlying molecular and cellular mechanisms. More recently, microphysiological systems (MPS), also known as “organs-on-chips” or “tissue chips,” leverage recent advances in cell biology, tissue engineering, and microfabrication to create *in vitro* models of human organs and tissues. MPS is a promising new approach methodologies (NAMs) that can model human physiology and disease *in vitro* and have multiple applications in areas where traditional cell culture and animal models fall short or are lacking. The National Center for Advancing Translational Sciences (NCATS) at the National Institutes of Health (NIH) who have been at the forefront in supporting the development of MPS have partnered with the International Space Station (ISS) U.S. National Laboratory, NASA and Center for Advancement of Science in Space (CASIS) in supporting the Tissue-Chips-in-Space program for biomedical research low earth orbit (LEO) to enable advances in the study of microgravity-associated human physiological changes. Over the past 7 years, the program has flown 15 various tissue chip payloads on 9 flight missions aboard Space-X to the ISS. Through this program, we have learned how microgravity exerts a unique range of stresses and pathophysiological perturbations resulting in dramatic increase in oxidative stress and inflammation, muscle wasting, immune senescence, cardiovascular deconditioning and cardiomyopathy, and alteration of gene expression. This program has provided information on human age-related dysfunction and age-associated diseases and underlying molecular mechanisms providing information for novel targets for drug development and innovative treatment modalities. A second iteration of this program is currently underway that will support development of complex multi-organ systems to better model physiological changes associated with aging-related functional decline and age-related diseases. Multi-organ platform integration will enable the study of multiple organ pathophysiology. Samples representing the broad spectrum of diversity in the human population will allow for better modeling of aging progression and development of interventions that consider disease heterogeneity and/or population diversity. The outcome and impact of the Tissue Chips in Space program for the MPS and NAMs field as well as the wider biomedical and health research communities will also be described.

Presentation: Oral

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Complement-Animal Research In Experimentation (-ARIE): A new NIH program to catalyze the development, validation and widespread use of new approach methodologies (NAMs)

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Scientific breakthroughs are often propelled by the introduction of new technologies that can transform how scientists study health and disease, and the 21st century has been a time of accelerated technological advancement. Increasing use of new and improved biomedical technologies, such as gene editing, artificial intelligence (AI), human induced pluripotent stem cells (iPSCs), and microphysiological systems (MPS) are fundamentally changing the way science is done. These advances serve to propel the development of new methods that can potentially reduce reliance on animal model systems and increase human relevance. These new approach methodologies (NAMs), provide a complementary approach to traditional models while offering tremendous promise for enhancing understanding of the human system towards more effective treatments and cures. Over the last 15 years, the National Institutes of Health (NIH) has increased investment in NAMs, including *in chemico*, *in silico*, and *in vitro* approaches, especially on MPS. To further catalyze and transform the use of innovative NAMs, the NIH Common Fund has initiated a new program, Complement-Animal Research In Experimentation (Complement-ARIE), which aims to catalyze the development, standardization, validation and use of combinatorial NAMs that will more accurately model human biology and disease states. This 10-year program aims to achieve the following goals: 1) better model human health and disease outcomes across diverse populations, 2) develop NAMs that provide insight into specific biological processes or disease states, 3) validate mature NAMs to support regulatory use and standardization, and 4) complement traditional animal models and make research more efficient and effective. To inform the implementation of Complement-ARIE, the NIH organized strategic planning events including an inter-agency retreat, public listening sessions, scientific literature landscape analysis, and a challenge prize. This US\$400 million program is currently being implemented through the establishment of a pre-competitive Complement-ARIE Consortium that will consist of 3 major components 1) Comprehensive NAMs Technology Development Centers (TDCs) that will stimulate the development of novel NAMs to address areas of greatest need, with emphasis on increased biological complexity and throughput, innovative combinatorial approaches, and data sharing, 2) NAMs Data Hub and Coordinating Center (NDHCC) that will create integrated data structures, including standards for model credibility, adherence to the FAIR (Findable, Accessible, Interoperable, and Reusable) principles of NAMs-relevant data, and create a searchable NAMs repository; and 3) Validation and Qualification Network (VQN) that will be a public-private partnership enterprise



that will establish common data elements and standardized reporting, apply validation and qualification frameworks, accelerate deployment and regulatory implementation of NAMs. Within each of these three key program components, training, community engagement, workforce development, and outreach activities will be integral to facilitate dissemination, capacity building, and adoption of NAMs. We anticipate these new methods and approaches could complement or, in some cases, replace traditional animal models, transforming the way we do basic, translational, and clinical sciences.

Presentation: Oral

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Mainstreaming animal-free innovation with transition management and helpathon interventions

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For a researcher to adopt new research models and take them further requires much more than relevant scientific knowledge. Making this transition demands other ways of thinking, doing and organizing research practices at the individual, institutional and national level. It requires practical implementation knowledge, knowledge of upcoming products and services, upcoming regulations and legislations, access to funds that invest in promising innovative research methods, personal skills to convince local research facilities to adopt a new research strategy and help them build the capacity to participate in local policy making.

To understand the dynamics of transitioning towards animal free innovation we adopted the transition management theory. Transition management has proven to be a good theoretical framework to describe and thereby get a sharp understanding on how dominant cultures, structures and practices work. It also provides a good understanding of how the dynamics of unusual innovative practices can grow into normal practices. It helps identify and develop new policies, mindsets and practical ways of doing things, to go beyond the status quo – technical knowledge and funds are not sufficient to create an environment of innovation. Transition management enables the proposers to assess what power different stakeholders have to enable the transition to animal free innovations. We discern the different types of power stakeholders have to understand their influence. For example, the status quo is kept by structures and procedures and people looking after them with assigned functions to do so. They have the power to keep up the mainstream. New possibilities can be explored by those who are intrinsically motivated to do so. They have the power to create.

In our helpathon practice we bring together both those who want to transition their practice and those who want to help others transition their practice together in a network organized around trust. Since 2017 over 300 researchers helped each other discover and adopt innovative animal free research models in a series of 10 helpathon venues. These are highly interactive workshops where participants volunteer to help researchers discover animal-free research possibilities. We helped researchers find grants to work with human tissues and apply or develop *in vitro* models. We helped them with publication strategies. We helped researchers break down their

research questions. We suggested alternative research strategies involving human data. We suggested researchers can become influencers. We involved relevant stakeholders (e.g., industries, regulators) to accelerate the development. We helped bring animal free education further.

We want to share our helpathon experience with you and demonstrate how helpathon interventions have the potential to introduce what these creators create into the mainstream. This challenging adoption of animal free innovations into the mainstream is exactly what is needed now.

Presentation: Oral

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Non-animal models in DIT testing

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Important progress has been made in the last decades in the field of *in vitro* immunotoxicology covering both immunosuppression and inappropriate immunostimulation. The possible application of *in vitro*, including the use of non-mammalian species, and *in silico* models in Developmental Immunotoxicology (DIT) research will represent a significant step toward more ethical, efficient, and accurate methods of assessing immunotoxicity risks. Research is focusing on improving the physiological relevance of *in vitro* models by incorporating 3D structures, organ-on-chip technologies, and human-derived cells to better replicate the *in vivo* environment. *In vitro* models have limitations, lacking the complexity of whole-organism responses, including interactions between different organs and systems, which should be carefully considered when addressing DIT. Stem cell-based models, 3D organoids, and co-culture systems represent innovative *in vitro* approaches for studying DIT. Stem cell-based models utilize pluripotent stem cells to investigate the processes of immune cell differentiation and function, providing valuable insights into how substances may affect the immune system at a cellular level. Similarly, 3D organoids are designed to mimic the structure and function of lymphoid organs, such as the thymus and bone marrow, allowing researchers to evaluate immune system development in a controlled environment. Co-culture systems further enhance these capabilities by combining multiple cell types to replicate the complex interactions between immune cells and surrounding tissues. Together, these advanced models offer powerful tools for studying immune system development and potential disruptions caused by chemical exposures. Validation for regulatory acceptance is a key challenge, requiring models to demonstrate reproducibility, reliability, and relevance through rigorous testing. Currently, validated *in vitro* methods to assess DIT are not available. As *in vitro* and *in silico* models continue to evolve, their combined use is expected to play a crucial role in DIT research. The status of the art of these models will be presented and discussed, including the models developed within PARC, a large EU project funded by Horizon H2020.

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Presentation: Oral



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A universally usable, xeno-free, defined medium for 2D and 3D culturing of normal and cancer cells

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Cell culturing methods are increasingly used to reduce and replace the use of live animals in biomedical research and chemical toxicity testing. Although live animals are avoided when using cell-culturing methods, they often contain animal-derived components, most commonly fetal bovine serum (FBS). FBS is added to cell culture media among other supplements to support cell attachment/spreading and proliferation. The safety, batch-to-batch variation and ethical problems with FBS are widely acknowledged [1] and therefore worldwide efforts are ongoing to produce FBS-free media.

Here, we present the composition of a new defined medium [2,3] with proteins derived either from recombinant sources or from human tissues: Oredsson Universal Replacement (OUR) Medium.

OUR medium supports long-term routine culturing of normal and cancer cells. Additionally, it can be used for cell banking and cryopreservation. We show growth curves and dose response curves of cells grown in two and three dimensions as well as applications such as cell migration. Cell morphology was studied in real time by phase contrast and phase holographic microscopy time-lapse imaging. OUR medium is successfully used with human normal and cancer-associated fibroblasts, human keratinocytes, human breast, pancreatic and colon cancer cells as well as several other normal and cancer cells [4,5].

In conclusion, we present the composition of OUR medium without animal-derived products that can be used for routine culturing and in experimental settings for normal and cancer cells, i.e., OUR medium provides a leap towards a universal animal-component-free cell culture medium.

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Presentation: Oral

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Case studies in animal-free science: Replacing antibodies and media in research

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To maximize the reliability of *in vitro* research methods, PETA Science Consortium International e.V. has focused on replacing the use of animal-derived components with their animal-free counterparts. These components include commonly used laboratory reagents, such as antibodies and ingredients in cell culture media. In the research sector, hundreds of suppliers produce millions of antibodies in addition to those developed by companies and academic institutions for internal applications. Millions of animals are estimated to be used to produce antibodies annually worldwide, and fetal bovine serum production for use in cell culture media uses upwards of 1.8 million bovine fetuses each year globally. To optimize *in vitro* research, the Science Consortium works to identify existing commercially available animal-free options and to develop new reagents when needed. This presentation will highlight several collaborative projects aimed at this goal, including a partnership with Abcalis and Unilever to develop recombinant animal-free IL-6 and IL-8 antibodies for ELISA applications using phage display technology and non-animal reagents. Additionally, the Science Consortium has collaborated with the Luxembourg Institute of Science and Technology to transition cell lines commonly used in inhalation toxicity testing to animal-free chemically-defined media. This presentation will describe the process of identifying animal-derived components and assessing the performance of alternatives, providing practical examples and demonstrating the feasibility of using existing technologies to make non-animal test methods truly animal-free, thereby adhering to ethical and legal requirements as well as the most reliable non-animal science.

Presentation: Oral



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The art of validation and peer review: Evolving practices while ensuring scientific confidence

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Method validation and peer review are indispensable components in the regulatory assessment of chemical hazards and safety. The principles of validation in a regulatory context were first established in the 1990s and gained international recognition with the adoption of OECD Guidance Document No. 34 (GD34) in 2005 [1]. GD34 also specifies that all test methods/approaches should undergo a formal and independent peer review at the end of validation, and prior to submission to regulatory authorities for acceptance. Even if the principles of validation and peer review remain relevant today and the process described in GD34 was successful in pioneering the regulatory acceptance of non-animal methods, an evolution of practices is needed to embrace emerging technologies (e.g., high throughput, artificial intelligence), the increasing complexity of the information measured (e.g., 'omics), and the need for data integration to address complex endpoints [2]. Moreover, as more validation studies are sponsored and managed by method developers, ensuring data integrity, transparency and independent review of the study is becoming paramount. Raw data and well-documented information supporting the method/approach's fitness for purpose, human biological relevance, and technical characterization should be accessible for review. Developers should be able to fund but not directly manage an independent review of their methods/approaches. It is also important to guarantee that members of peer review panels have no conflicts of interest to guarantee the independence of a review. This talk will give an historical perspective on validation and peer review and will provide insights into potential future directions to keep pace with scientific progress whilst ensuring scientific confidence and the protection of human and environmental health. Future directions in the regulatory context will involve adapting to technological advancements, fostering international collaboration, enhancing data transparency, embracing computational tools, and promoting inclusivity in the validation and peer review processes.

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Presentation: Oral

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Integrating all 3Rs using a scientifically-supported theory of behavior change

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The 3Rs are critical principles that should be used as a foundation to any research program that uses animals. However, although many research professionals have learned about the 3Rs briefly they may lack a depth of information on their implementation or not be supported in their efforts to apply the 3Rs in practice. This presentation will address the innovative strategy of the 3Rs Collaborative (3RsC) to focus on encouraging implementation of high-impact, practical, evidence-based 3Rs strategies by following a scientifically supported theory of human behavior change. This theory states that human behavior is guided by individual attitudes, social/professional norms, and self-efficacy. Grounded in this theory, the 3RsC first provides evidence supporting 3Rs strategies that target different values from science to animal welfare to cost. Then the 3RsC focuses on supporting practical implementation by providing resources, tips, and tricks to assist in the application of these techniques. Finally, we provide evidence of professional and social support for 3Rs techniques by collating logos, quotes, and stories of institutions that have switched. In addition to highlighting resources and efforts to create specific 3Rs changes, over-arching resources such as a virtual certificate course and institutional support for 3Rs and culture of care programs will be highlighted.

Presentation: Oral



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Towards the next generation of environmental risk assessment – The challenge of scalability

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Globally, environmental risk assessment (ERA) methodology is deeply rooted in the long-standing societal concern about the hazards of man-made chemicals and their potential risks to the environment.

Traditional ERA frameworks are based on laboratory-based hazard characterization data, typically combined with worst-case environmental exposure estimates. The methods used to develop these data have continuously evolved e.g., *in vitro* toxicity testing, ecological modelling, environmental fate modelling, satellite supported landscape mapping, and many others. Therefore, as we progress into the 21st century and further information types and quantity become available it becomes clear that combined with changes in societal expectations linked to broader information access e.g., via on-line sources, an updated risk assessment paradigm is needed.

This new ERA framework must be fit for contemporary challenges and expectations as the underlying societal drive for minimal unintended impact on human or environmental health and safety is increasing too.

The paradigm of risk assessment applied to chemical safety is changing. A next generation risk assessment paradigm is emerging, led by the cosmetics sector and focused on human safety. For environmental safety assessment application of NGRA (NGeRA), the human safety approach cannot be copied as the level of data and subsequent assessment for individual organisms needs to be extrapolated across vastly different and numerous species. Instead, the focus will be on creating models and frameworks that can be adapted and applied across various species and scenarios. This approach allows for a more scalable and efficient assessment process, ensuring that environmental safety standards are met without the need for exhaustive individual-level data for every species.

In this presentation we will highlight areas where scalability is of particular importance and propose some currently developed approaches that hold promise to facilitate the transition into a new era of NGeRA.

Presentation: Oral

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A human lymph node-on-chip with integrated lymphatics displays physiological immune properties

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To study systemic human innate and adaptive immune responses in detail, competent *in vitro* lymph node (LN) models with LN stromal cells (LNSCs) are required to recapitulate the physiological microenvironment. The multicellular organization of LNs possesses a challenge for designing such biological systems, particularly with the structural complexity of LNs and the lymphatic vasculature. Here, we aimed to culture an organotypic LN hydrogel model with integrated lymphatics, using an organ-on-chip (OoC) device containing a printed sacrificial structure, to study the influence of LECs on the LN microenvironment. Upon one-week culture under lymphatic flow, LECs lined the tubular structure forming a lymphatic vessel through the LN model, and stable metabolic conditions within the LN-on-chip were established. Interestingly, LECs in the LN-on-chip displayed the phenotype found in human LNs, with upregulation of LEC-specific LN markers, such as atypical chemokine receptor 4 (ACKR4). The addition of the LEC vessel to the LN-on-chip increased the total amount of native immune cells within the LN model, most notably B cells, and the secretion of more survival and migratory signals, namely interleukin-7 (IL-7) and CC motif chemokine ligand 21 (CCL21). Likewise, the lymphatic vessel promoted the abundance of immune cell clusters closer to the vessel, a fundamental cellular arrangement for efficient immune cell interaction. As such, the emergence of LN-specific features essential for a migratory microenvironment demonstrates the physiological relevance of a perfused lymphatic vessel. This approach provides the opportunity to integrate LNs into multi-OoC (MOC) platforms to investigate immunological crosstalk between tissue-derived factors, immune cell trafficking and organ-specific adaptive immune responses.

Presentation: Oral



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Introducing the ESTIV Early Career Network and its mentorship program

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The European Society of Toxicology In Vitro – Early Career Network (ESTIV ECN) was established in 2023/2024 to promote opportunities for early career scientists in the field of *in vitro* and *in silico* toxicology. In addition to a dedicated ESTIV ECN session at the biennial ESTIV Congress, the network provides various opportunities to enhance the soft (transferable) skills of emerging scientists.

One key initiative is the ESTIV ECN Mentorship Program, which will be introduced during the WC13 Congress. In its first edition, highly motivated early career scientists from academia and industry – at different career stages – were matched with senior scientists from various European countries based on their professional interests.

The primary goal of this initiative is to provide a unique platform for meaningful engagement and expert guidance at crucial career stages. Additionally, it serves as a catalyst for both personal and professional growth, helping early career researchers advance their careers, explore new opportunities, and maintain a healthy work-life balance. Further details on the program's structure and outcomes will be presented at WC13 to inspire young researchers attending the congress and to empower the next generation of scientists in the field of next-generation risk assessment.

Presentation: Oral

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Developing a toxicity testing platform for marine risk assessment using the tropical model amphipod *Parhyale hawaiiensis*

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Eco New Approach Methodologies (ECO-NAMs) applied to marine environments are of growing interest. Recently, invertebrates have been accepted under REACH requirement as alternative methods, e.g.,

bioconcentration in the freshwater amphipod *Hyaella azteca*. This encourages new opportunities for the development of ECO-NAMs using amphipods. There is a need for models representing the marine compartments. This work aims to introduce a testing platform using the amphipod *Parhyale hawaiiensis*. This species has a circumtropical distribution and inhabits coastal areas. Its genome has already been sequenced, and the species is well explored as a model in evolutionary developmental biology (EVO-DEVO) studies. They are easily cultivated in the laboratory in small chambers containing reconstituted seawater. In 2010, we started a culture of organisms collected from the field (coastal area in São Paulo state, Brazil). They are fed with fish food 4 times a week, maintained at 26°C, 12 h photoperiod, salinity of 30. Miniaturized acute and chronic assays were developed. In the acute test, neonates are exposed in 96 wells microplates for 96 h to evaluate mortality. The protocol was applied to several substances such as natural and synthetic dyes, metals (Cd, Cu, Zn, Ag), pesticides, 6PPD-quinone and aqueous environmental samples. To evaluate sediments, the test was adapted using 12 wells microplates. In the chronic assay, juveniles are exposed in 100 mL-vials for 42 days. This method was applied to assess zinc and 3,4-DCA effects on reproduction. Miniaturized genotoxicity protocols were also developed using adults in 12 wells microplates during 96 h exposure. The measured endpoints were micronuclei (hemocytes), DNA damage (comet assay – hemocytes and sperm cells) and cell viability. Hemocytes were characterized (total count and proportion of granulocytes, semi granulocytes and hyalinocytes) as well as their phagocytosis capacity, enabling the development of immunotoxicity protocols. Differential gene expression was explored after exposure to Ag nanomaterials opening interesting opportunities for molecular studies. A protocol to monitor embryos' development and abnormalities, after parental exposure, is being established. Toxicokinetic studies were performed with Ag ions and Ag-nanoparticles and Disperse Red 1 dye using hemolymph internal doses. Similar studies are currently being conducted for PFAS. For field studies, adult organisms (cultivated in the lab) were accommodated in cages (10 cm height x 20 cm diameter) and placed in sites with different levels of contamination. After 96 h, hemolymph and whole-body samples are collected and analyzed for chemical contaminants. *Parhyale* testing platform seems to be a powerful tool in ecotoxicology for both lab and field studies. We believe that *P. hawaiiensis* can be an attractive potential ECO-NAM for future risk assessment in tropical marine environments.

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Presentation: Oral



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Capturing complex biology is critical for accurate *in vitro* prediction of developmental toxicity

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Prenatal exposure to teratogenic compounds can lead to significant birth defects. Due to the high variation in drug response amongst species, and the financial and ethical burdens associated with animal testing, the development of advanced human cell-based assays is critical for effectively identifying and classifying potential human teratogens.

Over the past decades, assays such as TeraTox and the DevTox Germ Layer Reporter Platform (DevTox GLR) have used the directed differentiation of human induced pluripotent or embryonic stem cells (hiPSCs/ESCs) to assess the effect of chemical exposure on expression of early germ-layer genes as a measure for teratogenicity potential. These assay work as valuable tools for fast (2-7 days) high-throughput screening of compounds. Despite their good performance and contribution to the reduction of animal work, both assays have limited predictivity for the detection of embryotoxic compounds (60-75%). We have developed ReproTracker, an assay that follows the differentiation of hiPSCs into three germ layer cell lineages, ending in the development of specific cell types, namely hepatocytes, cardiomyocytes and neuronal cells. This assay is slightly longer (2 weeks) and can recapitulate a larger number of biological processes involved in early embryonic development.

In this study, we evaluated the effects of having a broader biological coverage and the ability of ReproTracker to offer more reliable teratogenicity predictions. To do so, we identified the teratogenic potential of a set of well-known *in vivo* teratogens and non-teratogens tested in the previously mentioned assays. Based on a set of 23 compounds, ReproTracker identified drugs misclassified by Teratox, including warfarin, methotrexate and ceritizine. Moreover, ReproTracker showed a higher accuracy (100% vs 76%) and sensitivity (100% vs 85%). Similarly, ReproTracker showed a higher accuracy and specificity (92%) when compared to DevTox GLR (accuracy 77% and specificity 83%) using a set of 27 compounds. Interestingly, most of the false negative compounds in TeraTox and DevTox GLR, affected tissue-specific biomarkers (AFP or MYH6), rather than earlier lineage markers, in ReproTracker, highlighting the relevance of biological coverage in teratogenicity assessment. These results emphasize the value of using a trilineage differentiation in the ReproTracker assay to provide a better recapitulation of multiple biological processes and to overcome teratogenicity detection limits observed in other assays.

Presentation: Oral

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Practical approaches for reducing and avoiding severe suffering

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Each year across the world, an estimated 10 million scientific procedures are carried out on animals with an actual severity of “severe”. This figure broadly represents around 10% of all animal research undertaken. Any level of suffering in animals used in research and testing is obviously a concern for everyone, but severe suffering is of greatest concern. There are both ethical and scientific imperatives to reach a point where no animal experiences severe suffering, and there is widespread support within the scientific community for working to achieve this.

Over the past decade, the RSPCA has been leading an important initiative involving the input of scientists, veterinarians, animal technologists, regulators, members of local animal ethics committees and others in the UK, European Union and internationally. The project identifies where (e.g., which animal “models” or tests) and why (reviewing scientific objectives and methods, current use of welfare assessment protocols and the implementation of humane endpoints. etc.) “severe” suffering occurs and has used collective expertise and insights to develop and promote practical approaches for reducing – or ideally, avoiding – it. In the UK alone we have seen year-on-year reductions, and an overall fall of 67% since 2014, in the number of experimental procedures causing “severe” suffering.

This presentation will highlight: the national and international events that have been facilitated by the RSPCA to bring together those involved in the regulation, care and use of lab animals to discuss the issue and share knowledge and ideas; the expert working groups and their reports focusing on providing practical actions and recommendations in specific research areas; and will also guide attendees through the practical “Roadmap for ending severe suffering” that individual institutions can follow to review opportunities for making progress in their own work.

Reference

www.focusonseveresuffering.co.uk

Presentation: Oral



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Optimization of safety assessment technologies using kidney microphysiological systems and organoids for animal-free testing

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Background and rationale: Traditional *in vitro* and *in vivo* models for renal toxicity assessment present significant limitations, including insufficient predictive accuracy and ethical concerns related to animal testing. Furthermore, the absence of comprehensive kidney-specific toxicity testing platforms impedes new drug development, especially for metabolic, immune, and cardiovascular therapeutics. This study addresses these gaps by developing advanced kidney-based alternative testing methods to enhance the predictive validity of renal toxicity assessments.

Methods: The research incorporates both kidney MPS and organoids to establish standardized protocols for renal toxicity evaluation. The study is structured in three phases:

- **Structural and Functional Characterization:** Kidney MPS were structurally verified using cell-specific fluorescent markers to ensure the accurate replication of renal architecture. Functional markers, such as CCK-8 assays, were used to assess cell viability and cytotoxicity in response to various compounds.
- **Assay Development and Optimization:** Different MPS models, including dynamic perfusion and static cultures, were evaluated to identify the optimal conditions for renal toxicity testing. Organoid-based toxicity testing included the evaluation of nephrotoxicity markers like NGAL expression and the use of multiplex immunohistochemistry (IHC) for detailed cellular responses.

- **Standardization and Validation:** Standard Operating Procedures (SOPs) were drafted and refined for both kidney MPS and organoid models. Cross-validation of protocols was performed across multiple laboratories to ensure reproducibility and reliability.

Results: The optimized kidney MPS demonstrated enhanced predictive capabilities for nephrotoxicity, with significant improvements in cell viability and structural integrity under optimized conditions. Similarly, kidney organoids exhibited high sensitivity to known nephrotoxins such as Cisplatin, Tacrolimus, and Gentamicin, with corresponding elevations in NGAL levels and other biomarkers. The cross-validation process confirmed the reproducibility of the developed assays, supporting their potential for broader application in drug safety assessment.

Conclusion: This study successfully optimized kidney MPS and organoid-based models for non-animal renal toxicity assessment. The standardized protocols developed herein provide a robust framework for predictive toxicology, offering a viable alternative to animal testing. These findings pave the way for regulatory acceptance of advanced *in vitro* models, promoting ethical, efficient, and accurate safety evaluations in drug development.

Presentation: Oral



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Addressing regional needs in Latin America: Overcoming barriers to NAM adoption for local tolerance testing

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The use of animals for product regulation is an important ethical and scientific issue across the world, the desire to reduce and replace is shared by most countries. Latin America is composed of 20 countries, including ranges from North, Central and South Americas, and parts of Caribbean. The legislation of these countries is different from each other, and most still use toxicological tests on animals for the registration of their products. In addition, the lack of technical knowledge on the part of the evaluators associated with the input costs for the new approach methodologies (NAMs) makes their use challenging in Latin America. Validated OECD guidelines are currently available for important toxicological endpoints such as genotoxicity, sensitization and skin irritation/corrosion, as well as eye damage. However, only 6 Latin American countries are part of the OECD Mutual Acceptance of Data (MAD) system, consequently another challenge to be overcome for the adoption of NAMs in this region. The first Brazilian federal law on the scientific use of animals was approved in 2008 (Law 11794), which created the National Council for Animal Experimentation Control (CONCEA), responsible for issued several normative using NAMs and recognizing it in the country, subsequently. The regulatory framework was in 2019, especially for agrochemical, when the National Health Surveillance Agency (ANVISA) published RDC 294. The current legislation does clearly refer to the use of NAMs approved by CONCEA, OECD, or authorities with similar regulatory exigences in article 6°. Therefore, NAMs are accepted for registering products at ANVISA, if performed following validated protocols. In view of this, Brazil is at the vanguard of Latin America regarding regulatory acceptance and use of NAMs. To further promote the adoption of NAMs throughout the region, collaborative efforts focused on knowledge sharing, technical training, and the development of region-specific guidance are crucial. Besides, moving forward, a concerted effort is needed to expand the OECD MAD system to include more Latin American countries and to provide accessible training and resources to regulatory bodies throughout the region. By prioritizing the implementation of validated NAMs, Latin America can align with global best practices, reduce reliance on animal testing, and promote innovation in product development, and consequently foster both ethical and scientifically sound regulatory practices.

Presentation: Oral

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ASPIS academy for sustainable toxicology through NAMs

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The evolution of chemical risk assessment has been marked by the emergence of New Approach Methodologies (NAMs), offering a transformative paradigm that replaces traditional testing approaches with more efficient and human-relevant methods. At the forefront of advancing NAMs for next-generation chemical risk assessment are Early-Stage Researchers (ESRs), whose development and support are critical for driving innovation in the field. Recognizing this critical need, the ASPIS Academy (AA) serves as a comprehensive networking initiative that brings together over 120 ESRs from three major H2020 European projects within the ASPIS cluster (ONTOX [1], RISK-HUNT3R [2], and PrecisionTox [3]). Through targeted initiatives encompassing training, mentorship, research group collaboration, laboratory exchange programs, outreach activities, and sustainability efforts, the AA is committed to fostering ESR professional development. These initiatives are coordinated by ESR representatives with guidance from experienced researchers. AA embraces diversity by fostering an inclusive environment that welcomes participants of varied backgrounds, beliefs, and identities, ensuring equal opportunities for emerging scientists to develop their ideas and aspirations. As a model for supporting early-career researchers, the AA demonstrates how to create an enabling environment while addressing challenges and pursuing continuous growth. This work advances the dialogue between AA and the broader community, demonstrating the contributions of ASPIS ESRs and illustrating the innovative capacity of emerging scientists.

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Presentation: Oral



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The ORCHESTRA project: Understanding how chemical mixtures shape reproductive health

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In an orchestra, each musician plays an instrument. Each instrument produces a unique sound. Traditionally, scientists have tried to understand how each of these instruments, or in this case, each chemical, affects human health individually. However, in real life, we are not exposed to a single “instrument,” but rather to a complex symphony of multiple instruments that can influence our health. This symphony represents the chemical exposome, which includes exposures throughout our lives, influencing our health.

The ORCHESTRA project resembles a new way of conducting the orchestra. Instead of listening to each instrument individually, it listens to the entire orchestra to understand how the combination of these compounds affects our health. The project evaluates a “musical mixture” of 50 endocrine disruptors identified in the European RiskMix Project and a local mixture (from Costa Rica), at a reproductive level using *Daphnia magna* and *Danio rerio* as models.

By studying chemical exposures as one would listen to a full orchestra – through *in vivo*, *in vitro*, and proteomics analyses – the ORCHESTRA project uncovers the intricate interplay of these chemicals and their collective impact on reproductive health.

Presentation: Oral

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Evaluating kinetics, metabolism, and toxicity in one *in vitro* system

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Replacing animal testing with new toxicological study methods particularly for endpoints that have historically been dependent on animal testing such as systemic toxicology is important. Understanding potential systemic exposure requires linking multiple organ systems and then tracking the parent compound, metabolites, and toxicity. This information can provide an improved understanding of human exposure and safety. Cinnamaldehyde is a fragrance ingredient whose metabolism and toxicity is well understood. Therefore, this material was selected as a case study. We used a human dynamic multiple organ platform that provides a means to determine *in vitro* kinetics, metabolism, and toxicity. For the current project, cinnamaldehyde was evaluated in a three-organ circuit (intestine, liver, and kidney). Commercially available *in vitro* systems were used to model the intestine, liver, and kidney, and communication between organs was modeled by a simulated blood flow system that incorporates a semi-permeable membrane inside each organ compartment. This system allows the test chemicals to move by osmotic diffusion into and out of the blood flow and organ compartments. Kinetic concentration versus time curves were created to observe ADME kinetics for the parent compound and its metabolite (cinnamic alcohol). Kinetic concentration versus time curves showed rapid intestinal absorption and distribution to liver and kidney. The primary metabolite, cinnamic alcohol, was also detected in the intestine chamber. Organ viability was measured by lactate dehydrogenase (LDH) release and by MTT reduction. In conclusion, this unique integrated organ platform has demonstrated metabolism, kinetics, and toxicity for cinnamaldehyde consistent with the literature. Our results provide a means of parameterizing the human *in vitro* microphysiological system (MPS) platform for building quantitative models designed to improve next generation risk assessment (NGRA).

Presentation: Oral



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The state of sex reporting in toxicology: A systematic review

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Despite well-documented sex differences in disease prevalence, progression, and therapeutic responses, preclinical toxicology research has historically exhibited a significant bias toward male subjects. This imbalance has contributed to critical knowledge gaps, leading to incomplete risk assessments and potential inaccuracies in chemical safety evaluations.

To investigate the extent of sex bias in toxicology research, we performed a systematic review examining the title and abstract of articles published between 1995 and 2025. This review assessed how the sex of both cellular models and animals was reported. Articles were categorized by publication year, journal, country, affiliation, sex of the first author, and the sex of the cells or animals utilized. Our analysis identified trends over time and highlights the persistent underreporting of sex in toxicology studies involving cellular models, with significant implications for translational research and regulatory guidelines.

These findings emphasize the urgent need for greater transparency and standardization in sex-based reporting within toxicology research. By recognizing and addressing the extent of these disparities, researchers and policymakers can develop strategies to better integrate sex as a biological variable in future studies. Such integration will improve the reproducibility of scientific findings, enhance the accuracy of chemical risk assessments, and ultimately contribute to the development of safer and more effective drugs.

Presentation: Oral

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Building confidence in MPS use in regulatory applications: The 3Rs collaborative's & FDA-CDER's cross-platform DILI project

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With expanding interest in greater integration of non-animal methods in drug development, there is great potential for human-centric, physiologically relevant liver microphysiological systems (MPS) to address gaps in preclinical testing, improving drug development and patient outcomes. However, to increase widespread integration of MPS in drug discovery and development there is a need to establish confidence in the accuracy, reliability, and standardized characterization of such methods. Only through increased confidence in their performance will there be wider usage, industry reliance on, and specific regulatory guidance for these platforms.

The FDA's Center for Drug Evaluation Research (FDA-CDER) and the 3Rs Collaborative's MPS Initiative (3RsC-MPS) are conducting a project under a consortium agreement with involvement from the US National Toxicology Program Interagency Center for the Evaluation of Toxicology Methods (NICEATM) and the Critical Path Institute (C-PATH). Its main goal is to perform an evaluation across 10 companies' liver MPS using standard conditions from a similar baseline protocol to evaluate drug induced liver injury (DILI) for a focused panel of hepatotoxicants using the matched endpoints of toxicity. The design and performance differences in each platform is expected to highlight the expected variabilities that might be observed in study reports, for those endpoints of interest in the prediction of DILI risk in humans. The choice to use liver MPS is based on their increasing use in drug discovery, and the fact that most MPS manufacturers have liver platforms which are considered relatively advanced in their development.

This project is expected to support increased confidence in the use of MPS across platforms and study sites. In turn, it may lead to broader adoption of MPS which will contribute to increased submissions of MPS-based studies in drug applications, and ultimately their greater integration in drug development and regulatory decision making. This will be a step towards the potential eventual replacement of animals for specific toxicological endpoints.

Presentation: Oral



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Something from nothing: How chemicals extracted from human blood act as mixtures in *in vitro* neurotoxicity

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Complex mixtures of organic pollutants are ubiquitous in the environment and humans. This vast array of man-made chemicals threatens human health even if each individual chemical is present below levels of concern, as they can interfere with biological processes and cause adverse effects as mixtures. However, most risk assessment and (bio)monitoring approaches focus on individual chemicals or distinct groups, such as plasticizers, pesticides, or flame retardants. This fails to account for the diversity of chemical mixtures and their mixture effects.

We propose an effect-based approach that combines chemical analysis with cell-based *in vitro* bioassays to quantitatively link mixture composition and bioactivity. This concept has been successfully applied in environmental studies, where chemicals detected in rivers and water bodies were linked to concentration additive responses in mammalian cell assays targeting bioactivity endpoints such as oxidative stress, endocrine disruption, and xenobiotic metabolism. More recently, we extended this approach to human samples as effect-based human biomonitoring (EB-HBM).

EB-HBM was applied in a case study on neurotoxic chemicals in the blood of women at 34 weeks of pregnancy from the German LiNA mother-child cohort. Developmental neurotoxicity was chosen as the endpoint, given concerns that prenatal chemical exposure may impact neurodevelopment in children, though the causative chemicals or mixture effects remain unknown. The selected high-throughput neurotoxicity assay, using differentiated SH-SY5Y neuroblastoma cells, measured interference with neurite development and nonspecific cytotoxicity.

An analytical screening method, coupled with an automated evaluation algorithm, targeted 1,012 chemicals in plasma samples from 624 pregnant women and detected and quantified 294 chemicals – spanning industrial chemicals, pesticides, pharmaceuticals, personal care products, food-related chemicals, and endogenous compounds. The measured exposure was directly linked to the neurotoxic effects of sample extracts. However, the predicted neurotoxic effect of detected chemicals with quantified neurotoxic activity explained only up to 0.31% of the experimental mixture effects in plasma extracts.

We demonstrated that mixture effects followed the principle of concentration addition by testing 78 designed mixtures composed of 61 chemicals in combinations of 4 to 28 components mixed in concentration ratios that were detected in the blood of individuals. For 93% of the artificial mixtures, predicted mixture effect deviated by less than a factor of two from the observed experimental effects. This experimental proof of global additivity at low effect levels supports the “something from nothing” phenomenon in human samples and health-relevant endpoints – where chemicals contribute to mixture effects despite occurring at concentrations too low to cause measurable individual effects. From a risk assessment perspective, this underscores the need to acknowledge the reality of chemical exposure as complex mixtures at low concentrations.

Presentation: Oral

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Accelerating the adoption of 3Rs approaches in biologicals testing

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Biologicals such as vaccines, cytokines, enzymes, and hormones are tested extensively post-licensure as part of routine quality control (QC) and batch release testing with an estimated 8 million animals a year used worldwide for this purpose. This animal use puts a significant financial burden on manufacturers and national control laboratories, is time and resource intensive, and the methods themselves can cause significant pain and distress to the animals. In contrast, appropriate *in vitro* methods are more scientifically relevant and can substantially reduce assay variability and the time and resources required.

With Bill & Melinda Gates Foundation funding we have reviewed the animal testing requirements in World Health Organization (WHO) guidelines for the QC and batch release testing of vaccines and biological therapeutics. A follow-on project, also funded by the Gates Foundation, will support delivery of the 3Rs impacts of our recommendations, driving their implementation and accelerating the adoption of the latest non-animal testing methods by the global biologicals community. Embracing the latest advances in replacement technologies will support faster access to cheaper vaccines by the communities who need them most urgently.

This presentation will highlight the key findings and recommendations from the WHO review, showcase key resources that NC3Rs has created to support the implementation of these recommendations and describe ongoing work to drive the adoption of 3Rs approaches in biologicals QC testing.

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Presentation: Oral



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Internal exposure characterization to identify relevant metabolites for chemical risk assessment

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In order to establish a robust NGRA, it is essential to provide an estimation of the internal exposure of the parent chemical and the relevant metabolites. The potential toxicological effects are determined through various toxicodynamic assays, facilitating quantitative risk assessment. The underlying hypothesis guiding the internal exposure assessment involved employing Physiologically based pharmacokinetic (PBPK) modeling to estimate the pharmacokinetic (PK) profile following relevant exposure scenario of a face cream containing 0.5% Benzyl salicylate (BSal). The absorption, distribution, metabolism and excretion (ADME) parameters for the modeling originated from *in vitro*/ *in silico* methods addressing skin penetration, major metabolite identification, skin and liver metabolism, and renal clearance. The most exposure-relevant metabolite Salicylic Acid (SA) which was generated due to fast liver cell metabolism (90% within 30 min) underwent testing to assess its potential toxicodynamic effects through pharmacology profiling, cell stress, transcriptomics, DART (ReproTracker) assays. This presentation describes a case study that highlights the importance of the combination between *in vitro* and *in silico* methodologies to have a robust internal exposure characterization and identify the relevant metabolites for chemical risk assessment, which guides the following toxicodynamic assays of these metabolites. The identification of the toxicologically critical entity is a critical step to guide the workflow and the selection of chemicals to be tested in toxicodynamic assays. Our evaluation showed that both the traditional risk assessment and NGRA identified the same major metabolite (salicylic acid) as the driver of toxicity. It could also be demonstrated that the *ab initio* approach is at least as conservative as the traditional approach for consumer safety.

Presentation: Oral

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Micropollutant mixtures quantified with *in vitro* bioassays and chemical analysis in surface water of Rio de Janeiro compared to global water pollution

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Background: A variety of organic micropollutants have been detected in aqueous matrices for decades, threatening ecosystems and human health, and chemical analysis alone cannot capture them comprehensively. Effect-based methods are reliable tools for assessing the effects of complex mixtures of chemicals in water quality monitoring and identifying the impacts of both known and unknown chemicals, when combined with chemical analysis.

Aim: To comprehensively assess *in vitro* biological effects of mixtures of organic micropollutants present in surface waters from Rio de Janeiro State, to identify main toxicity drivers and to benchmark effects against water quality worldwide.

Methods: Water from the Guandu River basin extracted with solid-phase extraction were investigated with *in vitro* bioassays comprising several target modes of action: xenobiotic metabolism through activation of aryl hydrocarbon receptor (AhR CALUX), pregnane X receptor (PXR) and peroxisome proliferator-activated receptor gamma (PPARγ GeneBLAzer), hormone receptor-mediated effects with estrogenic (ERα GeneBLAzer) and glucocorticogenic (GR GeneBLAzer) activities, developmental neurotoxicity (neurite outgrowth inhibition with differentiated SH-SY5Y cells) and oxidative stress response multiplexed with mitochondrial membrane potential (MMP) inhibition in AREC32 cells (MitOxTox). Cytotoxicity was assessed in parallel for every bioassay. Water extracts were also analyzed for 611 chemicals with target screening using liquid chromatography high-resolution mass spectrometry (LC-HRMS). Measured effects were compared with results obtained worldwide with similar bioassays and benchmarked against effect-based trigger (EBT) values in a risk-based approach.

Results: A total of 227 chemicals were detected in surface waters extracts, many of which are pharmaceuticals or pesticides stemming from WWTPs or tributaries. The bioassays revealed that the mixture of micropollutants can trigger effects *in vitro* that are likely to cause adverse effects in the environment and impact the quality of the main regional source of drinking water. ERα activation was the most concerning endpoint and primary risk driver, but general cytotoxicity, neurotoxicity and AhR activation should also be considered with moderate risk to aquatic ecosystems. The observed effects were poorly explained by quantified chemicals through iceberg modeling, highlighting the impor-



tance of combining chemical analysis with bioanalytical tools to evaluate mixture effects and indicating that many unknown chemicals contribute to the mixture effects. Nevertheless, some toxicity drivers could be identified, such as 1,2-Benzisothiazolinone, Mebendazole, Diuron, Diclofenac, and 2-Benzothiazolesulfonic acid. The *in vitro* effects of micropollutant mixtures observed in the present study are comparable to those of human-impacted sites examined in different regions of the world. ERα, followed by AhR activation, were the most concerning endpoints in every continent, whereas other endpoints were less frequent and less representative from a risk perspective.

Conclusion: The effects observed in surface water extracts from a crucial water source in Rio de Janeiro align with the current global context of pollution, where mixtures of micropollutants are pervasive in aquatic environments worldwide. However, it remains essential to address other endpoints and unmonitored regions in future monitoring efforts.

Presentation: Oral

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Molecular similarity and property cliffs

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Molecular similarity is a key concept in molecular design and optimization. It is the basis of the similarity principle, which states that similar compounds have similar properties. The concept has been broadly used in biomedical investigations developing bioactive compounds for drug discovery applications. Representative applications are virtual screening and predictive modeling, including the prediction of toxicity. A notable exception to the similarity principle is activity cliffs or, more generally, property cliffs, which are defined as pairs of compounds with high structure similarity but very high and unexpected activity. Activity cliffs provide key information to optimize properties and pose significant challenges in property prediction. Despite the broad applications of similarity, precisely defining similarity is still challenging, largely in part due to the subjectivity of the concept. In this work, we discuss the role of molecular similarity in drug design, chemical space exploration, molecular representations, and toxicity prediction.

Presentation: Oral

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Towards eco-safe cosmetics: Accurate reef-safe claim validation with the RGB method

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When developing sunscreens, prioritizing both human efficacy and environmental safety is critically important, particularly with regard to the protection of coral reefs. Previous research has identified potential adverse effects of specific cosmetic ingredients on corals. However, the absence of standardized testing protocols poses a significant technical challenge to formulating coral-safe sunscreens. To address this, and in adherence to ethical guidelines aligned with Directive 2010/63/EU and Brazilian Resolution nº 58/2023, this study proposes a novel New Approach Methodology (NAM) for assessing the acute toxicity of sunscreens on corals.

This study tested twelve sunscreen formulations with varying Sun Protection Factors (SPFs) by applying them to fragments of the coral *Seriatopora hystrix*. Fragments, approximately 3 cm in length and containing over 100 polyps, were exposed to different sunscreen concentrations for 48 and 96 hours. Visual assessments of polyp retraction and coral bleaching were conducted, along with the determination of the Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) for each sample.

Quantitative RGB (Red, Green, and Blue) color pattern analysis was performed using Photoshop 23.5.1 to reinforce visual evaluations. An increase in RGB value ranges, with a maximum of 765, corresponded to greater coral bleaching. Statistical analyses using TOXSTAT 3.5 supported the interpretation of the results. Of the twelve products evaluated, eleven showed no observable adverse effects on coral fragments, as evidenced by the absence of significant polyp retraction or bleaching, and this was corroborated by stable RGB values. Notably, one product caused polyp retraction and coral bleaching, a finding confirmed by significant deviations in RGB patterns when compared to the initial state.

These findings highlight the importance of a holistic approach to addressing the complex interactions between cosmetic formulations and coral health. Instead of simply excluding controversial ingredients like oxybenzone and octinoxate, this study emphasizes evaluating the complete product composition to ensure more reliable assessments of a sunscreen's impact on coral reefs.

In conclusion, this study introduces a NAM for evaluating sunscreen toxicity on corals, enabling the classification of "reef-safe" products. The methodology provides a practical framework for developing sunscreens that balance environmental protection with consumer needs, supporting efforts to safeguard marine ecosystems and promote sustainable practices in cosmetic science.

Presentation: Oral



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Multiorgan-on-chip models for replacing animal models: Lung-gut axis models to better mimic human pathophysiological responses for testing safety, efficacy, and superiority of new therapeutics in lung disease and injury research

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Organ crosstalk is fundamental to physiology and pathophysiology, making multi-organ animal replacement systems essential for evaluating the safety and efficacy of drugs, chemicals, and nanomedicines.

For example, chronic obstructive pulmonary disease (COPD), the third leading cause of death worldwide, has treatments that are largely supportive. Developing reparative lung treatments requires reliable models that mimic human pathophysiology. Communication along the lung-gut axis is crucial for maintaining and controlling organ functions, making combined lung-gut models essential. Given COPD's link to inflammation and the gut's critical role in immunity modulation, integrating gut and immune system models with lung models is necessary.

We established two classes of lung-mimicking models: healthy and diseased lungs (COPD). The COPD model emulates human disease characteristics, including dysfunctional cellular barrier function, depletion of ciliated cells, and overproduction of goblet cells. These models offer competitive advantages over existing *in vitro* lung models, such as using patient-derived cells for personalized medicine, an extracellular matrix protein interface for physiological cell adhesion and differentiation, and media microcirculation to mimic dynamic lung conditions. Additionally, these models enable the use of inhalation/aerosol devices used in human clinical trials, facilitating research translation.

Our gut model employs an intestinal triple culture system, incorporating Caco-2 and HT29-MTX-E12 cell lines with fluidic flow to simulate physiological conditions. Impedance and transepithelial electrical resistance measurements evaluate barrier formation and integrity. The model is further developed into healthy and inflamed states by incorporating differentiated THP-1 macrophages with and without inflammatory stimuli. This model assesses drug effects on gut health and their downstream effects on lung function.

The final model assembles both lung and gut systems, providing a comprehensive platform for testing new therapeutics and understanding complex organ interactions.

Presentation: Oral

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Fostering a community of young scientists in non-animal approaches: The EURL ECVAM Summer School experience

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The European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) organizes a biennial Summer School on Non-Animal Approaches in Science, tailored for post-graduate students and early-career scientists. The Summer School aims to share knowledge on the latest non-animal methods and technologies, promote their use in biomedical research and regulatory applications, and explore career paths in non-animal approaches. Since its inception in 2017, the Summer School has trained over 400 students worldwide. An impact survey conducted among alumni revealed a significant influence on their current research or career paths, with many contributing to the progress of non-animal approaches in their professional activities. The Summer School has created a community dedicated to advancing alternatives to animal testing and promoting ethical and innovative scientific practices. This year, EURL ECVAM will host its fifth Summer School, with a record-breaking number of applications received, exceeding 300. Notably, the 2025 edition will also feature students participating as part of the Student Ambassador Project. This represents a new initiative aiming to disseminate knowledge and raise awareness about non-animal approaches among university students, fostering a network of student ambassadors who can share information with their peers, and inspire new generations to develop, use, and teach about them. This presentation will provide an overview of the Summer School's objectives, activities, and impact, highlighting its role in shaping the future of non-animal methodologies in science.

Presentation: Oral



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Using NAMs to evaluate microcystin hepatotoxicity and potential carcinogenicity

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Natural toxins produced by cyanobacteria constitute a chemically diverse family, with microcystins (MCs) among the most concerning due to their frequent occurrence in cyanobacterial blooms, which are projected to increase with climate change. MCs are cyclic peptides with approximately 310 known congeners and are recognized for their hepatotoxic risks to human health. However, toxicological data are primarily available for MC-leucine arginine (MC-LR), the most extensively studied congener, which exhibits tumor promotion activity and is classified as possibly carcinogenic to humans (Group 2B). Given the substantial data gaps regarding MC toxicity, we employed multiple approaches to address this issue. Specifically, we evaluated the applicability of read-across for assessing the tumor promotion effects of data-poor MC congeners based on structural similarity and mode of action (PP2A inhibition as the key initiating event), in accordance with scenario 4 of the ECHA Read-Across Assessment Framework (RAAF), which anticipates quantitative variations in effects elicited by source and target substances via a common mechanism. This exercise demonstrated high structural and physico-chemical similarity between MC-LR (source) and 17 congeners (targets e.g., MC-LA, MC-RR), along with toxicodynamic similarity supported by bridging studies. The read-across for tumor promotion effect in the liver from MC-LR to other MCs was scored as “acceptable with just sufficient confidence”, and uncertainties related to experimental data quality and gaps in mechanistic insights for both target and source compounds constrained the confidence level of this read-across. To address these gaps and elucidate the mechanisms underlying hepatotoxicity and potential carcinogenicity, we developed *in vitro* studies with three MC congeners exhibiting differences in cellular uptake, detoxification efficiency, and structural characteristics (minor amino acid position variations). Transcriptomic data (mRNA, miRNA) from MC-LR, MC-LA, and MC-RR in the Hep-

aRG model demonstrated that, despite all three congeners exhibiting similar PP2A inhibition, MC-LR and MC-LA clustered together based on hepatotoxicity- and carcinogenicity-associated gene expression profiles, whereas MC-RR showed a response pattern more similar to the control (untreated). Furthermore, differences in proliferative effects among these MC congeners were observed in the HepaRG model, with a significant increase detected for MC-LR and MC-LA but not for MC-RR. This finding raises the hypothesis that PP2A inhibition alone may not fully account for MC-induced effects in the liver. To further investigate this, computational approaches, including molecular modeling and simulation analyses, have been employed to elucidate dynamic interactions between MC congeners and PP2A, as well as their potential interactions with other phosphatases and kinases. Additionally, metabolomics studies on MC-LR in the HepaRG model were conducted to elucidate hepatotoxic mechanisms through metabolite dynamics over time and concentration-dependent effects. Overall, data gaps in MC toxicity have been addressed through New Approach Methodologies (NAMs), with applicability domains defined in each case to enhance confidence in the hazard assessment of this class of natural toxins.

Presentation: Oral

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Accelerating the uptake of non-animal methods in cardiovascular research: An integrated approach

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The use of animal models in cardiovascular research is a critical area of focus for improving the implementation of the 3Rs (Replacement, Reduction, and Refinement), as mandated by Directive 2010/63/EU. A key step towards this goal is the analysis of Non-Technical Summaries (NTS) from the ALURES database, which provides valuable insights into current animal use in research. Building on this analysis, the European Commission will be conducting a thematic review on the implementation of the 3Rs in cardiovascular research, aiming to accelerate the uptake of non-animal methods and approaches.

We conducted an in-depth analysis of NTS focusing on cardiovascular research projects. This analysis, which classified over 3000 NTS based on criteria such as investigated pathology, species involved, and predicted severity of harm, revealed key trends and areas for improvement in the use of animal models. Building on this analysis, we are leading the European Commission's first thematic review, as mandated by Directive 2010/63/EU. This review aims to accelerate the uptake of non-animal methods and approaches in cardiovascular research and help both project submitters and evaluators. It will integrate our NTS analysis with other resources, including our previous biomedical review of non-animal methods available or in development in cardiovascular research, a new AI-based database of non-animal methods (BioMed-ModelHub), and stakeholder feedback. The thematic review will identify opportunities and challenges for using non-animal methods, analyze their utility and potential for wider application, and promote dialogue and collaboration within the cardiovascular research community. By combining these approaches, we aim to produce actionable scientific knowledge and foster the development of more human-relevant, non-animal models for cardiovascular research.

Presentation: Oral

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Elucidation of adverse outcome pathways for compound-induced developmental neurotoxicity using knowledge graphs

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Developmental neurotoxicity (DNT) is a common adverse outcome of early-life exposure to harmful compounds. Understanding the causes, nature, and complex molecular mechanisms underlying compound-induced DNT requires analyzing disparate knowledge sources. Knowledge graphs (KG) have emerged as a powerful means of harmonizing heterogeneous data sources and revealing non-obvious mechanistic connections linking compounds, genes, and biological processes.

We have developed a specialized DNT-KG by combining and harmonizing curated DNT data from several established data sources to capture known associations between genome-wide interactions, compounds, and their potential to induce DNT. The DNT-KG comprises over 1.3 million nodes, 1.5 million edges, and 8811 unique compounds and integrates the harmonized assay information from the EPA CompTox Dashboard, Alliance of Genome Resources, Clinical and Environmental Exposures Service (ICEES), and the National Institute of Environmental Health Sciences (NIEHS) owned DNT Data Integration and Visualization Enabling Resource (DIVER). Thus, the DNT-KG contains compound-gene-protein annotations, compound-protein interactions, gene ontology classifications, and human-animal gene ortholog mappings.

DNT-KG is publicly accessible and offers an interactive platform to elucidate Adverse Outcome Pathways (AOPs) for compound-induced DNT by KG mining. Querying DNT-KG for mechanistic AOPs linking compound exposure and DNT can identify respective molecular initiating events (MIEs) and key events (KEs). We illustrate this approach by identifying and constructing AOPs for 29 developmental neurotoxicants that appeared to be false negatives in existing *in vitro* assays. The investigation revealed MIEs and KEs not detected by current testing frameworks. Future investigations will capture information from the AOP-wiki to enhance inferences in line with current Organisation for Economic Co-operation and Development (OECD)-endorsed DNT AOPs.

In summary, DNT-KG is a novel resource for exploring knowledge concerning the molecular mechanisms underlying compound-induced DNT. This resource offers researchers data and tools to generate and explore DNT AOPs. The tool also enables researchers to identify and prioritize underlying key biological events that require developing new mechanistic assays. Thus, in alignment with initial OECD recommendations, the DNT-KG holds the potential to support the development of a battery of *in vitro* assays that can be employed as New Approach Methods (NAMs) for regulatory decision support concerning compound risk assessment.

Presentation: Poster



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Expanding the applicability domain: Filling data gaps to enhance the predictability of mutagenicity of synthetic and natural dyes using miniaturized alternative test systems

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Dyes are necessary in modern life and are used in innumerable products e.g., cosmetics, textiles, pharmaceuticals, food and paper. Many present mutagenic properties, but a significant number have not been evaluated. There are still major data gaps in the literature that prevent the development of hazard prediction tools with accuracy. Due to their different properties, many existing prediction tools (e.g., pharmaceutical-based QSARs) do not adequately cover the applicability domain of dyes. Traditional mutagenic tests to increase the amount of data describing the hazard of dyes usually require great amounts of samples. Our aim is to present an alternative approach to characterize the mutagenic properties of dyes using miniaturized assays to inform both human and environmental health. In this work, we will present the data generated from a promising strategy to fill data gaps and enhance existing and future predictive tools: the combination of a miniaturized version of the Ames test (Microplate agar, MPA), which detects point mutation, combined with a miniaturized micronuclei (MN) test for the detection of chromosome mutations, using the invertebrate *Parhyale hawaiiensis*. This combination aims at reducing reliance on vertebrate *in vivo* models, with the goal to increase available data for animal-al-

ternative approaches in the future. We analyzed 12 dyes with > 95% purity. For natural anthraquinone dyes (emodin, alizarin, dermocybin, and dermorubin), 5 synthetic (four non-commercial disperse dyes from the Max Weaver Dye Library, NCSU, US and C.I. Direct Blue 218). As an approach to removing the mutagenicity of emodin, we synthesized and tested 3 derivatives (allyl ether and mono and di acrylate esters). Less than 50 mg of each compound was sufficient for both test systems. MPA is performed in 12-well plates with and without metabolic activation (S9). We employed six strains of *Salmonella* (YG1041, TA1537, TA98, TA97a, TA100 and TA102) in a tier approach. MN was scored in the hemocytes for the detection of chromosome mutations. Among the natural dyes, only emodin and alizarin were mutagenic in the MPA and in the MN. Dermocybin and dermorubin, were negative in the MPA, and are still being analyzed for MN. The two ester derivatives of emodin were mutagenic in the MPA and MN, but the allyl ether derivative was negative in both assays. We believe that the former results arise from the conversion of the ester derivatives to emodin during S9 activation. In the case of the synthetic dyes, three of the non-commercial disperse azo dyes were mutagenic both in the MPA and MN, and one was mutagenic with MPA but not in the MN. C.I. Direct Blue 218 was negative for MPA but positive for MN. The combination of the Microplate Agar (MPA) and the MN test with the invertebrate *P. hawaiiensis* can be a powerful new method to characterize mutagenicity of dyes already in use or under development, especially when small quantities are available for testing. We believe this testing strategy can be expanded to cover more compounds and generate the necessary data to accurately reflect the applicability domain. It will enhance the predictability of existing hazard assessment models and the development of new models for compounds that are underrepresented, such as dyes. This will help in the future to reduce and refine the need for *in vivo* models in the assessment of dye mutagenicity for both human and environmental health.

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Presentation: Oral



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Ensuring transparency and rigor in the peer review of new approach methods for skin sensitization assessment

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As new methods to assess chemical safety are developed, independently conducted validation and peer review panels provide the necessary oversight and guidance to confirm that a new method is robust, reliable, relevant, and reproducible. Historically, these efforts have been conducted under the oversight of validation organizations such as those overseen by government bodies of the European Union, the United States, and Japan. These organizations conduct comprehensive validation and review processes under the guidance of a validation management team and peer review panel. However, historic approaches often fail to keep pace with the rapid development of new methods. To address this need, the Organization for Economic Cooperation and Development (OECD) has called for increased validation funding and expanded activities outside of the validation organizations. Without proper structure and oversight, this could result in the absence of a formalized validation management team and peer review panel. Given the need for a comprehensive peer review process to assure validity of new methods, this raises questions of how to support an independent peer review without creating a conflict of interest associated with the funding source, particularly when the validation is funded by the test developer. In this presentation, we will discuss the design, conduct, and lessons learned from a first-of-its-kind test method developer-funded peer review. We will focus on how the peer review panel approached the initial request, designed a transparent process, and conducted the peer review while ensuring independence of the panel and the peer review report. The talk will highlight areas where steps could be streamlined to increase efficiency of the panel and document review, including recommendations on timing of data audits, packet completeness assessments, validation document review and updating, and formal development of the report.

While in this review the panel maintained its independence and transparency, there was the potential perception of a conflict of interest given that the review was funded by the test method developer. These perceptions required the review organizers to plan carefully to alleviate independence concerns. This aspect of the review will be the focus of a discussion on a proposed new paradigm for peer review. Throughout this talk and after, participants in the session will have opportunities to address questions raised by the case study and discuss future directions and ideas to both improve the efficiency and clarify the appropriate approaches to support new ideas around formal validation that can be adopted by OECD and regulatory authorities.

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Presentation: Oral

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Integrating NAMs for quantitative skin sensitization potency assessment into regulatory frameworks: Experience from the work in OECD TG 497

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Understanding the potential for skin sensitization hazard and potency of chemicals is required for their acceptance and use. This need applies to a range of consumer use products and agricultural and industrial chemicals, all having different regulatory contexts. As animal testing is being limited or eliminated globally, the need for harmonized nonanimal testing approaches to address these endpoints has increased. The Organisation for Economic Cooperation and Development (OECD) develops test guidelines that fulfill these needs. These guidelines fall under the OECD principle of Mutual Acceptance of Data, a system that ensures that quality data produced within one country is accepted in other countries subscribing to the system. OECD Guideline 497, "Defined Approaches on Skin Sensitisation," published in 2021 was a first-of-its-kind guideline that provided three defined approaches (DA) for predicting skin sensitization hazard and potency categorization under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals. DAs apply fixed data interpretation procedures to data generated with a defined set of information sources and derive predictions without the need for expert judgment. Recently, OECD has undertaken the evaluation of skin sensitization models that can predict a quantitative point-of-departure (PoD) for inclusion in Guideline 497, adding an additional endpoint to the guideline. These models can provide human-relevant PoDs that can be subsequently applied within regulatory risk assessment frameworks. This presentation will provide a brief outline of the process by which new test guidelines are evaluated by OECD for development, using Guideline 497 and its ongoing expansion as a case study. The specific focus of the discussion will be OECD evaluation of new DAs for inclusion in a test guideline, using the Skin Allergy Risk Assessment – Integrated Chemical Environment (SARA-ICE) DA as an example. The presentation will also consider how to utilize this quantitative PoD in a subsequent risk assessment.

Acknowledgements: This project was funded in whole or in part with federal funds from the NIEHS, NIH under Contract No. HHSN273201500010C. The views expressed above do not necessarily represent the official positions of any federal agency.

Presentation: Oral



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Predicting points-of-departure and potency categories using new approach methods: Practical experience from the fragrance industry

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Dermal quantitative risk assessment (QRA) is essential for determining safe concentration levels of potential skin sensitizers in consumer products. The fragrance industry has developed a QRA approach, now updated to QRA2, in which the No Expected Sensitization Induction Level (NESIL) serves as the starting point or point of departure (PoD). Typically, the NESIL is a No Observed Effect Level (NOEL) in $\mu\text{g}/\text{cm}^2$, confirmed through human studies known as “Confirmation of No Induction in Humans.”

As confidence in NAM-based quantitative potency assessments grows, both animal testing and confirmatory human testing for PoD derivation may be eliminated. Several NAMs assess quantitative potency, including Bayesian models like SARA and SARA-ICE, the GARDskin dose-response assay, and OECD *in vitro* regression models. Studies have been conducted to evaluate the quantitative potency predictions of fragrance ingredients using the GARDskin dose-response and OECD regression models.

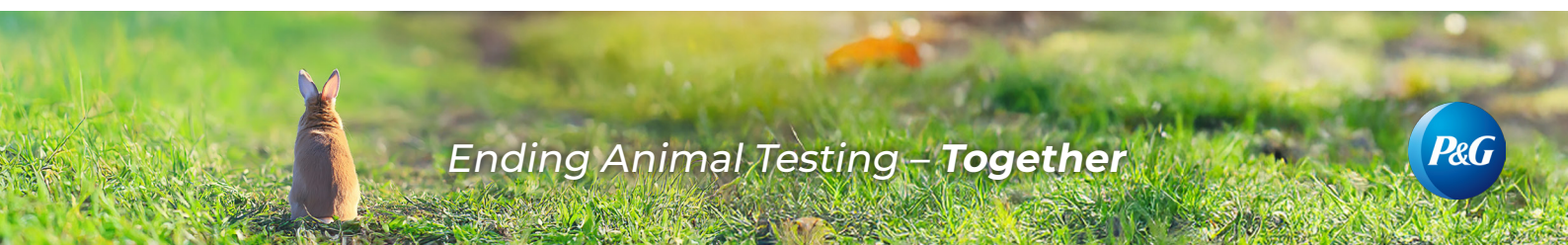
The GARDskin Dose-Response (DR) assay is an adaptation of the validated GARDskin assay (OECD TG 442E), providing a quantitative potency prediction similar to the NESIL value. An evaluation of the GARDskin DR assay’s precision and reproducibility for fragrance ingredients has been published. The predicted potency values were consistently reproducible and showed a strong correlation with the refer-

ence dataset. In addition, the assay’s application in QRA for fragrance materials within a Next Generation Risk Assessment (NGRA) framework has been demonstrated. The dataset was expanded to include a wider range of fragrance ingredients with varying structural reactivity and potency, resulting in the testing of 100 materials. Results were categorized into sensitization potency levels: extreme, strong, moderate, weak, very weak, or non-sensitizer. Potency categories were predicted with 37% accuracy, and approximate accuracy (exact match or one category off) was 81%. Combining predicted weak and very weak categories, increased total accuracy to 53%, and approximate accuracy rose to 98%. The mean prediction error for NESIL and Local Lymph Node Assay (LLNA) EC3 values was 3.15- and 3.36-fold, respectively.

Quantitative regression models based on OECD *in vitro* assays have been developed to calculate PoDs for use in skin sensitization QRAs. These models predict PoDs as Local Lymph Node Assay (LLNA) EC3 values (EC3) or human potency values (PVs), using data from *in vitro* assays. As with the GARDskin DR analysis, results were categorized into sensitization potency levels. Fold differences between predicted EC3s and PVs and experimental EC3s and human NOELs were calculated. Additionally, concordance between predicted and experimental weight-of-evidence (WoE) categories was assessed. It was found that the distribution of EC3 and PV categories closely mirrored the published WoE categories. High concordance was found between predicted PoD values and WoE categories. For example, the combined KS and kDPRA regression model achieved a 50% exact match, 41% off by one category, and captured 91% of all materials assessed. The median fold-misprediction for models predicting EC3 values ranged from 1.8 to 2.5, while for models predicting PVs, it ranged from 1.7 to 3.4.

Based on these results, both the GARDskin dose-response assay and *in vitro* regression models show strong potential as predictors of skin sensitization quantitative potency, increasing confidence in their use for Next Generation Risk Assessment.

Presentation: Oral



Our Commitment to #BeCrueltyFree



Our Animal Free Safety Science
(New Approach Methodology, NAM)



Learn More Details About Our NAM Science: DPRA
(Direct Peptide Reactivity Assay for Skin Allergy Safety) and Read-Across



Our Animal Welfare Achievements



Our Animal Welfare Advocacy Assessing Safety Without Animal Testing: The Road Ahead; George P Daston, Catherine Mahony, Russel S Thomas, Mathieu Vin ken; Tox Science 2022, Vol 187, p214-8.



Ending Animal Testing For Good Regulatory Change Needed to Unleash Animal Free Safety Science; Espe Troyano & Harald Schlatter, Politico June 13, 2023



Better Protection of People and our Planet While Building a World Without Animal Testing - Together
Patrick Masscheleyn Euractive 2020



Examining animal testing for risk assessment:
A WC-12 workshop report



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The development of animal-free alternatives to coral toxicity testing using symbionts

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Coral reefs support exceptional marine biodiversity and provide essential ecosystem services; however, the frequency of bleaching events and coral mortality has increased. Among the various stressors associated with these events, chemicals have been identified as significant contributors to the decline of coastal coral reefs. Consequently, identifying potential chemical pollutants is crucial for reef conservation. However, this process presents several challenges, including accurately determining the intrinsic hazard properties of chemicals to corals and establishing cause-and-effect relationships between coral responses and chemical exposure over time. Therefore, we have explored the use of coral symbionts in developing alternative methods for coral toxicity through case studies with ingredients of cosmetics, specifically those belonging to the categories of UV filters (11), preservatives (2), and emollients (1), since they can potentially reach marine environments, including coral reefs sites, through untreated wastewater discharges and recreational activities. Among the symbionts, we have focused on the potential use of Symbiodiniaceae dinoflagellates (zooxanthellae) and alterations in the coral microbiome. Six different species of zooxanthellae, differing in their functional roles within symbiotic interactions (photoprotection, carbon fixation, nitrogen fixation, thermotolerance, phosphate and nitrate assimilation, and coral larval protection), were cultured under controlled conditions ($22 \pm 2^\circ\text{C}$, 14 h photoperiod, light intensity of $45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and their absolute growth curves were determined to verify their potential use as test species. Five species presented adequate growth rates for growth inhibition tests and were then used to evaluate the toxicity of the cosmetic ingredients over a 72-hour test period. EC50 values derived from growth response curves did not show a common response pattern across species; instead, the results demonstrated species-specific variations in chemical sensitivity, suggesting that a multi-species approach may be required to accurately predict the chemical toxicity for zooxanthellae. The effects of the selected cosmetic ingredients on coral microbiomes were assessed through metagenomic analysis (16S rRNA gene sequencing) of non-exposed (control) and 96-hour exposed coral fragments (*Seriatopora hystrix*). The treatment effects on the microbial community vary among the cosmetic ingredients. While some ingredients have minimal effect on the microbiome, others induced changes in microbial richness and the presence of specific taxa previously associated with coral dysbiosis. These findings highlight the importance of evaluating the potential hazards of individual cosmetic ingredients; however, the broader consequences for coral health and product-level effects remain to be fully elucidated. More-

over, hazard information on individual cosmetic ingredients helps foster responsible product development activities within the cosmetic industry. Overall, our findings are expected to contribute to the advancement of marine ecotoxicity. New approach methodologies (NAMs), with a particular focus on hazard identification of chemical substances in corals.

Presentation: Oral

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Meta-analysis as a tool in the application of the 3Rs

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Since 1959, with the publication of Russell and Burch, the 3Rs principle (reduction, refinement, and replacement) has guided animal research and, more recently, served as the theoretical foundation for legislation related to animal experimentation in different countries.

There are many publications and high investment in producing basic science studies involving animals. However, in recent years, it has become clear that, as in other areas of research, the reproducibility rates of these articles are low, and few results are confirmed in subsequent clinical trials. Additionally, it is estimated that most results from animal studies are not published, leading to publication bias in the available literature. It is common to find studies with low statistical power, lack of randomization, and blinding during execution, insufficient attention to the design of the experimental setup, among other issues. In this context, we can now consider that, beyond the 3Rs related to animal welfare, we should also consider the need for the 3Rs related to methodological quality (robustness, registration, and reporting) (Strech and Dirnagl, 2019).

In preclinical meta-analyses, by analyzing all articles aimed at answering a specific research question, it is possible to identify divergent results in the literature and analyze the published data collectively, ensuring greater robustness of the result. Thus, it is possible to draw conclusions on a specific topic without the need to use new animals, contributing to the replacement and reduction. Furthermore, considering that during the development of the preclinical meta-analysis, there is a stage of assessing risk of bias or reporting quality, the execution of this technique itself offers a critical perspective that allows for the appreciation of studies with higher methodological quality and refinement of the experimental design for new studies to be conducted with animals.

The results of a survey with Dutch researchers (Luijk et al., 2011) and from a workshop held with researchers, members of the Animal Ethics Committee, and members of the Animal Welfare Committee (Luijk et al., 2012) pointed to the use of preclinical systematic reviews and meta-analysis as a potential tool to enhance the implementation of the 3Rs.

The introduction of the Systematic Review and meta-analysis methodology in animal research can lead to improvements in scientific quality and animal welfare, as well as a greater implementation of the Three Rs.

Presentation: Oral



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Understanding the concept of similarity and its applications to toxicological research and risk assessment

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The field of toxicology is undergoing a paradigm shift as computational approaches, particularly those involving New Approach Methodologies (NAMs), are becoming increasingly central to the prediction and assessment of chemical toxicity. While toxicologists have historically relied on traditional wet-lab techniques, the growing need for more efficient, ethical, and predictive methods in risk assessment has led to the rise of computational toxicology and cheminformatics. Central to the success of these approaches is the concept of chemical similarity, a concept that forms the foundation for clustering and classification approaches (CCAs) in toxicology. This presentation will explore the critical role that chemical similarity – both structural and biological – plays in advancing toxicological predictive capabilities.

The presentation will begin by defining chemical similarity and its importance in the context of computational toxicology, emphasizing that similarity is not a uniform concept and can vary significantly depending on the specific goals of a study. Chemical structures include molecular features (e.g., functional groups, ring structures), that play a central role in understanding how similar chemicals may interact with biological systems. By comparing these features and linking them to toxicological or biological activity profiles, we can gain insights into their potential effects.

A critical distinction that will be explored is the difference between endpoint-agnostic and endpoint-specific similarity. Endpoint-agnostic similarity refers to the general comparison of chemicals based on broad chemical features without pre-defined biological or toxicological outcomes. This approach is commonly used in unsupervised methods, which group chemicals as clusters without any prior knowledge of specific toxicological endpoints. Unsupervised methods are particularly useful in exploratory studies, allowing researchers to identify novel patterns, generate hypotheses, and explore chemical space. However, these methods are not well-suited for making definitive predictions about toxicity, as they do not account for specific biological responses. In contrast, endpoint-specific similarity focuses on the relationship between chemicals and specific toxicological outcomes, enabling the development of predictive models through supervised methods. Supervised learning techniques allow for the grouping of chemicals into classes of known or predicted biological activities. These methods are highly effective in chemical assessments, but they require careful management to avoid biases, such as overfitting or the inappropriate extrapolation of findings to new chemicals. Applying unsupervised methods in endpoint-specific contexts, such as read-across analyses, can lead to misleading conclusions if general chemical similarities are mistaken for relevant toxicological similarities. Both forms of similarity are instrumental in the analysis of chemical structures, but they must be applied appropriately based on the research context.

For toxicologists, understanding these nuances is vital for ensuring the proper application of similarity-based methods in toxicology. The pres-

entation will delve into the practical implications of these concepts for toxicologists, highlighting how balanced approaches combining both unsupervised and supervised methods can also be effective.

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Presentation: Oral

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The CATMoS model: An *in silico* tool for predicting acute oral toxicity

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A wide variety of chemical substances must be evaluated for their potential toxicity to ensure their safety for both human health and the environment. Acute oral systemic toxicity testing underpins regulatory decisions related to hazard classification, labeling, and risk management. However, the traditional rodent acute toxicity tests required for evaluating new and existing chemicals are costly and time-consuming. *In silico* models offer a promising alternative, enabling rapid predictions of acute toxicity without the need for animal testing.

To address this challenge, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Acute Toxicity Workgroup facilitated an international collaboration aimed at developing *in silico* models for predicting acute oral toxicity across five key endpoints: Lethal Dose 50 (LD50), U.S. Environmental Protection Agency hazard categories, Globally Harmonized System for Classification and Labeling hazard categories, very toxic chemicals (LD50 ≤ 50 mg/kg), and nontoxic chemicals (LD50 > 2,000 mg/kg).

A comprehensive dataset of acute oral toxicity data for 11,992 chemicals was created, divided into training and evaluation sets, and shared with 35 international research groups, which collectively submitted 139 predictive models. These models were validated using external validation sets and combined into consensus models to enhance predictive accuracy and maximize coverage across the chemical universe.

The resulting Collaborative Acute Toxicity Modeling Suite (CATMoS) integrates the strengths of individual models to deliver robust and accurate predictions. CATMoS has shown high performance in comparison to *in vivo* results and is currently being assessed by regulatory agencies as a potential substitute for *in vivo* rat acute oral toxicity studies. With predictions for over 800,000 chemicals available through NICEATM's Integrated Chemical Environment, CATMoS is also accessible through the free, open-source OPERA tool, enabling predictions for new and untested chemicals. This collaborative approach marks a significant step toward more efficient, ethical, and scalable human health hazard assessments.

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Presentation: Oral



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Assessing the utility of the Collaborative Acute Toxicity Modeling Suite (CATMoS) from ecological risk assessment to human health hazard

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Humans are exposed to thousands of chemical substances requiring assessment for their potential toxicity to ensure public health and safety. Acute systemic toxicity testing, which forms the basis for regulatory hazard classification, labeling, and risk management, is a critical aspect of these evaluations. Traditional rodent-based acute toxicity tests, while informative, are costly, time-consuming, and raise ethical concerns due to their reliance on animal testing. *In silico* models, leveraging existing data to predict toxicity without the need for animal use, provide a more efficient, cost-effective, and ethical alternative for assessing acute toxicity.

To advance this alternative approach, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Acute Toxicity Workgroup initiated an international collaboration aimed at developing *in silico* models for predicting acute oral toxicity across five key endpoints: Lethal Dose (LD50), U.S. Environmental Protection Agency (EPA) hazard categories, Globally Harmonized System for Classification and Labeling (GHS) hazard categories, very toxic chemicals ($LD50 \leq 50$ mg/kg), and nontoxic chemicals ($LD50 > 2,000$ mg/kg). A comprehensive acute oral toxicity data inventory, comprising 11,992 chemicals, was compiled and split into training and evaluation datasets. This data was shared with 35 international research groups, who collectively submitted 139 predictive models. These models were rigorously validated against external validation sets, and the resulting predictions were synthesized into consensus models, aimed at maximizing accuracy and chemical space coverage by leveraging the unique strengths of individual approaches. The resulting suite of models, the Collaborative Acute Toxicity Modeling Suite (CATMoS), demonstrates high accuracy and robustness, outperforming *in vivo* test results in many cases. This initiative is especially important for both ecological risk assessment and human health hazard evaluation, as it provides a more comprehensive framework for predicting toxicity in a range of chemicals relevant to both fields.

As a result, CATMoS has gained recognition from regulatory agencies for its potential to replace traditional *in vivo* rat acute oral toxicity studies. A recent collaboration with the US EPA compared LD50 values predicted by CATMoS to empirical values from *in vivo* studies for 177 pesticide active ingredients and found the predictions to be highly reliable, particularly for nontoxic substances. The models are integrated into NICEATM's Integrated Chemical Environment (ICE) tools and datasets, offering accessible, real-time predictions for a wide range of chemicals. Moreover, CATMoS has been implemented in the free and

open-source tool OPERA, allowing predictions for untested chemicals. This accelerates both regulatory decision-making and safety evaluations, enabling the identification of potentially harmful substances.

Ultimately, the development and application of CATMoS are a significant step in the transition from traditional animal testing to more advanced, computational approaches. This collaborative model provides a valuable tool for human health hazard assessment, streamlining the identification of toxic substances while reducing reliance on animal testing and promoting more sustainable, ethical practices in toxicity evaluation.

This research was supported [in part] by the Intramural Research Program of the NIH.

Presentation: Oral

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Utilization of non-animal approaches to support pesticide assessments in the United States

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The United States Environmental Protection Agency (U.S. EPA) regulates the use of pesticides and establishes maximum levels for pesticide residues in food under broad authority granted in two major statutes – the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act. To support registrations, regulatory statutes allow the U.S. EPA to require data and relevant information from pesticide registrants. This includes numerous studies that can require a large number of animals for testing. However, the regulatory statutes also provide U.S. EPA with flexibility to utilize all available toxicity and exposure information to consider alternative approaches, such as data waivers, read-across/bridging, and application of new approach methodologies (NAMs). These approaches provide opportunities for EPA to reduce its reliance on animal testing and improve its evaluations through the incorporation of more species relevant information. EPA is also actively engaged with multiple national and international organizations and stakeholders on efforts to develop and implement strategies that move away from traditional animal toxicity testing, while also meeting its mission of protecting human health and the environment. This presentation will provide an overview of these efforts and examples of how the U.S. EPA Office of Pesticide Programs have utilized non-animal approaches in a weight of evidence approach to support pesticide assessment.

Presentation: Oral



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Leveraging structural and biological similarities to fill data gaps – Read across supported by NAMs

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Read-across is one of approaches used for assessing systemic toxicity for cosmetic ingredients, especially in Europe where animal testing is banned. When relevant data-rich analogues are identified, it is straightforward to derive a point of departure from the analogues after ensuring similarity on structural and functional properties. When the analogues identified are data-poor, one possibility is to fill the data gaps with new approach methodologies covering both toxicokinetic and toxicodynamic properties. A ten-step process for read-across supported by new approach methodologies was used for this case study. We used this workflow to evaluate Octisalate, a commonly used ingredient in cosmetics and personal care products. Our hypothetical situation was a gap in systemic toxicity data for octisalate used at 5% in as a sunscreen. In the first tier, a systemic exposure dosage was calculated using the European Scientific committee for consumer safety's notes of guidances (2023). After gathering physicochemical data on octisalate, analogues were searched based on structure and organic functional groups using open source tools such as the OECD QSAR TOOLBOX v4.2, ChemTunes v1.2 and ChemID. Thirty-four analogues were identified that were later reduced to four when repeated-dose legacy *in vivo* data and *in silico* alerts relevant to systemic toxicity were considered. Like the target compound, all four analogues were predicted to release salicylic acid as the main metabolite. After collecting *in silico* and existing absorption and metabolic data, homosalate and cyclohexylsalicylate were the closest analogues, which have alerts on reproductive toxicity and hepatotoxicity. The margin of safety obtained using homosalate was less than 100 and refinement of the assessment is needed. Overall, the similarity between octisalate, homosalate and cyclohexyl salicylate were confirmed. The measured ADME properties were used to input a PBK model (Gastroplus 9.8.2) and an internal concentration (C_{max}) was calculated for octisalate: 0.004615 µg/mL. Using a legacy study for the analogue, a rat plasma concentration of 0.348 µg/mL was calculated using a PBK model (Gastroplus 9.8.2) with *in silico* input parameters. A margin of internal safety was calculated: C_{max} rat/C_{max} consumers = 75. Given this is an internal margin of safety a threshold of 25 is protective of human health and demonstrating that new approach methodologies can be used to fill gaps in read-across. This is an incremental step towards animal-free assessment of systemic toxicity where NAMs are used to fill data gaps and increase confidence in read-across where legacy data from analogues are not sufficient for decision-making. Building on published use cases, a best practice document might foster the widespread use of read-across enhanced by NAMs.

Presentation: Oral

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A roadmap to phase out animal testing: Moving beyond the science

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In 2023, the European Commission adopted a communication setting out its response to the European Citizens' Initiative: "Save Cruelty Free Cosmetics – Commit to a Europe Without Animal Testing." One aspect of that communication included a commitment to work with all relevant parties on a roadmap towards chemical safety assessments free from animal testing. The roadmap will serve as a guiding framework for future actions and initiatives to reduce and ultimately eliminate animal testing in the context of chemicals legislation within the European Union. However, it may also serve as a template for similar initiatives elsewhere, thus it is important to consider the essential elements for a roadmap to end animal use for chemical safety assessments and more broadly, e.g., biomedical research and education and training.

Creating a comprehensive roadmap to end the use of animals in safety assessments involves a multi-faceted approach integrating scientific, regulatory, policy, ethical, and practical considerations. However, while roadmap-related discussions and activities often focus on technological innovation, successful implementation hinges on strategic change management. Effective change management in this context requires addressing regulatory inertia, industry hesitancy, and public perception alongside significant shifts in practices and infrastructure while simultaneously navigating multi-stakeholder issues such as scientific validation and cross-sector collaboration. As such, investing significant resources in establishing a governance system will be necessary to achieve the desired goal.

One aspect of a successful transformative governance system includes incorporating specific goals and milestones to measure progress over time, ensuring that short- and long-term objectives are met. Key indicators to measure progress may include building on established baselines and metrics regarding animal use but should also include tracking wider objectives such as the regulatory acceptance of non-animal methods, funding, education and training initiatives, communication strategies and steps to enhance global harmonization and acceptance of methods. This presentation will discuss how such indicators may provide valuable insights into the effectiveness of change management strategies and highlight areas that require refinement. Ultimately, we can only achieve a future free from animal testing by looking beyond scientific progress and incorporating structured change management approaches with clear metrics.

Presentation: Oral



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Developing African origin neural organoids as a genetically diverse pre-clinical model of neurological disease

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In recent years there has been a rapid development of stem cell-based models of neurological development. These model systems, collectively known as brain organoids, have generated much interest for their ability to overcome the limitations of other models, particularly the use of rodents, have the potential to be the definitive system within which to study neural development and disorder. Brain organoids are generated as 3D *in vitro* aggregates of human neurological tissue derived from human pluripotent stem cells that can model the human brain microenvironment. Critically, cerebral organoids can be derived from induced pluripotent stem cells (iPSCs), allowing for the possibility of generating patient specific brain organoids and represents a powerful approach to study the genetic, proteomic, and structural changes caused by neural disease pathogenesis without the need for animal models. In the context of critical brain health challenges, particularly those faced on the African continent, brain organoids present as a uniquely accessible and physiologically relevant model system within which we can develop crucial insights into the disease pathogenesis and potential treatment options for brain diseases. Despite their immense potential, cerebral organoids, have yet to be developed in any African university or institution and, critically, never from iPSCs of African origin.

Our research focuses on generating African origin iPSCs and using them to develop novel models of neurological development. We make use of various signaling molecules and ligands and investigate how they drive anterior differentiation in iPSCs. We show that Wnt signaling has a posteriorizing effect on stem cells and directs differentiation towards the mesoderm, as confirmed through the upregulation of posterior and mesodermal markers. N2 and B27 can mitigate these effects and drive anterior differentiation. Critically we find that BMP4 signaling as a pulse can drive neural differentiation, while signaling centers result in better recapitulation of aspects of anterior-posterior axis formation with polarized expression of primitive streak markers as well as both anterior and posterior genes being upregulated. With the immense genetic diversity within African populations and the important role this can play in both disease progression and treatment there is significant scope to develop African specific brain organoids to investigate brain disease and development. Our findings are the first steps in generating diverse brain organoids that can be used as a preclinical model of neurodevelopmental diseases.

Presentation: Oral

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Characterizing genetic susceptibility to chemicals for human and non-human organisms: Opportunities for data reuse

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Pollution is the third leading driver of biodiversity loss, and chemical impacts on within-species genetic diversity threaten the resilience and adaptation potential of different species. However, methods for assessing chemical impacts on the genetics of wild populations are limited and primarily depend on sequencing large numbers of organisms in a given geographic region, which is impractical for all chemicals and species/populations. Therefore, it is essential to build predictive methods that can harness existing lab and field data and make use of new available data in the future. Further, we propose that methods and data to characterize chemical impacts on both human and non-human organisms can mutually enhance the respective fields of human health and ecotoxicology/wildlife conservation.

We previously developed a data-driven method to assess potential mechanisms of genetic susceptibility to chemicals in humans by integrating publicly available data on pesticide activity, single nucleotide polymorphisms (SNPs), and nervous system diseases. Here, we explore the potential to harness these same data integration approaches using lab data from model organisms for human health, but to characterize chemical impacts on wild populations, both from the perspective of genetic susceptibility and potential adaptation. In addition to starting from lab toxicology data, we explore the potential for repurposing existing field data that has been generated in studies independent of chemical pollution (e.g., studies of population genetic structure). As a proof of concept, we used mice (*Mus musculus*) as a species with ample lab data generated for human health toxicology and wild boar (*Sus scrofa*) as a species with published field data generated to assess population structure.

For mice, we integrated datasets of chemical activity from the Comparative Toxicogenomics Database, pathway data from the DAVID Knowledgebase, and phenotype data from the Monarch Initiative using gene enrichment. Through this, we built 407,446 Chemical – Pathway – Phenotype linkages that may describe the mechanisms underlying chemically-induced adverse outcomes in mice (linkages for 2,709 total chemicals). In these linkages, we characterized SNPs that may modulate these chemical – adverse outcome relationships (starting from 7.6 million SNPs for lab mice in Ensembl) to predict drivers of genetic susceptibility and adaptation in wild mouse populations. For wild boar, we related a published dataset of SNP variation at different sampling sites in Europe (original study did not consider chemicals) to maps of modelled pesticide concentrations from the literature using a gene – environment association method. Of the 40,700 wild boar SNPs included in the analysis, 2,357 were significantly associated with pesticides (86 total pesticides). From here, we identified enriched Gene Ontology (GO) terms for these SNPs/genes and found 41 GO terms enriched, including several nervous system-related phenotypes (e.g., neuron to neuron synapse). Because many pesticides are known to have neuroactivity (e.g., malathion, diquat), this provides support for our predicted associations. These two analyses of mice and wild boar demonstrate the value of repurposing existing datasets and using complementary human health toxicology and ecology methods to assess the potential impact of chemicals on the genetic variation of wild populations.

Presentation: Oral



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The blurry benchmark: Variability and interpretation of *in vivo* toxicity data

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A great deal of focus in toxicology has shifted toward evaluating the suitability of NAMs for assessing chemical safety, and accompanying this shift, there has been a reflection on the quality and variability of guideline data used for decades as the basis for regulatory decisions. In many cases, *in vivo* test guidelines were initially validated using very few reference chemicals or not validated at all. Yet, results of *in vivo* tests are often used to benchmark NAMs by compiling results from animal studies without considering the reliability of those data. Examples of chemicals tested more than once in a *in vivo* guideline experiment are limited. Where available, analyses indicate results are discordant for 26% to 34% of chemicals in bioassays evaluating relatively simple endpoints (e.g., the uterotrophic, Herberger, and local lymph node assays).

There is interest in developing NAMs for complex endpoints, however, identification of reference chemicals to evaluate the performance of NAMs may be complicated because the *in vivo* guidelines include multiple endpoints. Furthermore, *in vivo* guideline tests for specific types of toxicity (e.g., reproductive or developmental neurotoxicity) also include measures of general, systemic toxicity. In such studies, the observed critical effect level is often a non-specific endpoint such as liver or body weight change. Non-specific *in vivo* effects may occur at lower doses or be concomitant with specific types of toxicity and thus, be difficult to resolve. While chemical safety decisions based on systemic responses from *in vivo* guideline tests may be effective for protecting against adverse responses, particularly when safety factors are applied to ensure exposures are below doses causing adverse responses, there is not an assumption that these non-specific effects are predictive for similar responses in humans or wildlife.

As we work toward a framework for evaluating chemical safety with less reliance on animal testing, it is important to consider how animal tests were historically evaluated and used to support regulatory decisions to avoid setting impossible standards for NAMs. The performance of NAMs is generally evaluated against *in vivo* reference chemicals, which has several consequences. First, NAMs cannot be expected to predict *in vivo* effects at a level that is higher than the reproducibility of the *in vivo* method. Second, in the absence of rigorous expert review, chemical tested in animal studies may be erroneously associated with effects on specific targets or organ systems which studies were designed to test and used to benchmark predictive NAMs. Lastly, the existing framework of toxicity testing that relies on animal studies is largely protective of human health rather than predictive of specific effects. Reconsidering NAM adversity in this context would substantially alter how NAMs are used and benchmarked.

Presentation: Oral

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The IATA framework template to standardize and build scientific confidence in complex NAMs

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Global trends in toxicology are shifting towards increased use of New Approach Methods (NAMs), however, complex endpoints generally require multiple NAMs methods used in combination. The OECD Integrated Approaches for Testing and Assessment (IATA) Case Study Project was designed as a forum for sharing experiences and building confidence in NAMs used in combination in a regulatory decision context. In addition, the project aims to consider aspects of IATAs that can be standardized, and when suitable, can lead to Defined Approaches (DA) which can be included in OECD Test Guidelines.

The OECD IATA Case Studies Project is now in its 11th year, and while a variety of Case Studies have provided examples of IATAs for complex toxicological endpoints, available DAs are limited to relatively simple, well-understood acute toxicity endpoints. To move away from a one-and-done approach of the IATA Case Studies and move towards increasing standardization, the IATA Framework Template was developed as a modular workflow that can be easily reused, updated, or modified. The IATA Framework includes a description of the purpose (regulatory relevance and context), chemical and biological domain, scientific basis underlying the IATA, the workflow and modules included to address the regulatory context (e.g., PBK, exposure, environmental fate and transport), information sources within in each module, data selection/evaluation criteria, and the approach for combining information sources (i.e., the data interpretation procedure). In addition to being a standardized reporting template, the IATA Framework is designed to be adaptable for different problem formulation. The same Framework Template can be used for more than one regulatory decision contexts (e.g., prioritization, hazard identification/characterization, tiered testing, quantitative PoD, exposure-led NGRA), which are linked to the workflow and specific modules included, and data interpretation procedure which provides an output suitable to address the decision context. In this way, the same information sources can be used (sometimes, with additional information) for multiple purposes, allowing IATAs to be easily reused.

Following the example of the recent OECD QSAR Assessment Framework (QAF), an IATA Assessment Framework (IAF) was developed to help evaluate the level of confidence in each element reported in the IATA Framework Template. Like the QAF, the IAF is intended to be a systematic and harmonized framework for regulatory assessment of IATAs. For each section of the IATA Framework Template, assessment elements were defined, along with criteria for evaluating the level of confidence and the degree to which each element is met, and the level of confidence in the overall IATA. This work is intended to support transparent and consistent approach for evaluating fitness of the IATA for use in regulatory decision making, increase the standardization and uptake of in IATAs that address complex endpoints, and function as a roadmap for building DAs. As experience is gained with IATA Frameworks and assessment of the approaches for complex endpoints, some mature



examples may be suitable for development as OECD guideline DAs. For IATAs that are not ready or suitable for inclusion in guidelines, this work may nevertheless increase the confidence and uptake of well developed, well documented IATAs that can be acceptable for supporting regulatory decisions.

Presentation: Oral

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Regulatory perspective on animal testing for cosmetics in Brazil

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The Brazilian Health Regulatory Agency (Anvisa) is responsible for regulating and monitoring the compliance of cosmetics in Brazil. The word “cosmetics” is used here to refer to cosmetics, personal hygiene products and perfumes. Resolution RDC 907, of 2024, from Anvisa defines and establishes technical requirements and procedures for the regularization of cosmetic products, among other requirements. This regularization must be done through registration or prior communication by notification. The company responsible for the regularization of cosmetic products must have complete data proving the safety of the product and ensure compliance with current regulations. However, it is only when proof of specific safety is required by current regulations or when a safety attribute is expressed on the label, that it is mandatory to present a summary of data proving safety of use in the product regularization process. The Guide for Safety Assessment of Cosmetic Products (2012) and Resolution RDC 35 (2015), which provides for the acceptance of alternative methods of animal experimentation recognized by the National Council for the Control of Animal Experimentation (Concea), published by Anvisa, establish general guidelines for Regulatory Acceptance of Non Animal Methods (NAMs) for Cosmetics in Brazil. Furthermore, publications from the International Cooperation on Cosmetics Regulation (ICCR), of which Anvisa is a member, and the Organisation for Economic Cooperation and Development (OECD), are also used as complements to Anvisa publications. Vertebrate animal testing is not required to assess the safety of cosmetics. It is possible to prove the required toxicological endpoints based on ingredient information, *in vitro* methods and/or clinical tests, following the Integrated Approaches to Testing and Assessment (IATA). The presentation will give an overview of the Regulatory Acceptance of NAMs for cosmetics in Brazil, considering the publications mentioned in this summary, and highlight the advances with the review of the Guide for Safety Assessment of Cosmetic Products, which is being carried out in 2025.

Presentation: Oral

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Regulatory acceptance of alternatives to animal testing for sanitizing products in Brazil

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The Brazilian Health Regulatory Agency (Anvisa) is responsible for regulating and monitoring the compliance of sanitizing products exposed to consumption in Brazil. The term “sanitizing products” is used here to refer to cleaning products, disinfectant and sanitizing products and insecticides, repellents and rodenticides. Resolution Anvisa RDC 59, of 2010, regulates the procedures and technical requirements for the notification and registration of sanitizing products. The notification and registration of sanitizing products are carried out taking into account the assessment and management of the risk, purpose and category of the product. Companies that manufacture sanitizing products are responsible for ensuring the quality, effectiveness and safety of their products and their regularization with Anvisa. On the other hand, Anvisa prioritizes efforts in product regularization activities aiming to reduce the use of tests on vertebrate animals. In 2014, the National Council for the Control of Animal Experimentation – Concea/Ministry of Science, Technology and Innovation, through Resolution 18, recognized 17 alternative methods to the use of animals in Brazil. Concea Resolution 54, of 2022, updated and revoked Resolution 18. Concea maintains an updated list of recognized alternative methods on its website. In 2015, Resolution Anvisa RDC 35 provides for the acceptance of alternative methods of animal experimentation recognized by Concea and establish general guidelines for Regulatory Acceptance of New Approach Methods (NAMs) for products subject to health surveillance in Brazil. In the regularization of sanitizing products, NAMs recognized by Concea are accepted, however, with restrictions for tests on insecticide, repellent and rodenticide products and for the evaluation of new active ingredients. Integrated Approaches to Testing and Assessment (IATA/OECD) that combine multiple sources of information are accepted to verify the toxicological parameters of sanitizing products. The presentation will give an overview of the Regulatory Acceptance of NAMs for sanitizing products in Brazil.

Presentation: Oral



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MFDS's progress in new approach methodologies (NAMs) and their regulatory acceptance in Korea

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Ministry of Food and Drug Safety (MFDS) has provided support for the promotion of NAMs since they were not widely popular nor extensively studied by, for example, conducting research and providing education on the methods in Korea. MFDS is the only government agency in Korea that has developed NAMs adopted in OECD Test Guidelines, etc. (four OECD TGs, one SPSF in progress, one standard planned for listing in ISO).

With the revision of Cosmetics Act in 2016, MFDS has also banned the sales and marketing of cosmetics and their ingredients tested on animals in Korea. The current Korean laws relating to NAMs are: Laboratory Animal Act; Cosmetics Act; Regulation on Evaluation of Functional Cosmetics; and Standards for Biological Safety Evaluation of Medical Devices, etc. MFDS is responsible for establishing and implementing policies to support development and acceptance of NAMs as an alternative to animal testing. Accordingly, the Minister of MFDS has established the Korean Center for the Validation of Alternative Methods (KoCVAM) that oversees development, validation, and dissemination of NAMs since 2009. Recently, MFDS has revised the Korean Pharmacopoeia to accept NAMs for quality control of pharmaceutical products. With the revision, test methods using recombinant factor C have newly been included to replace the use of horseshoe crab blood to detect endotoxins in pharmaceutical products. Also, MFDS has issued an administrative notice on implementation of the revised "Regulation on Pharmaceuticals Approval, Notification and Review". The revised regulation allows submission of data from non-animal methods or human biology-based tests (cell-based test, MPS, bioprinting, computational model, etc.) that are produced in accordance with Good Laboratory Practice.

Since 2024, the National Institute of Food and Drug Safety Evaluation (NIFDS) has been conducting a five-year research project titled, "Standardization of alternative test methods for practical implementation". The project aims to improve coherence of regulatory science and to encourage practical use of NAMs (organoid, MPS, 3D tissue, etc.) by developing, optimizing and standardizing NAMs for safety, efficacy and quality evaluation of drugs. MFDS, as a regulatory body, has focused on development and international harmonization of safety assessment methods using organoid.

To proactively adapt to the global expansion of NAM applications beyond cosmetics and medical devices to pharmaceuticals, MFDS has dedicated the past 16 years to advancing NAMs in Korea. With the expertise and experience accumulated through KoCVAM, MFDS is well-positioned to take a leading role both nationally and internationally in the field of NAMs

This research is supported by a grant (25211MFDS001) from Ministry of Food and Drug Safety in 2025.

Presentation: Oral

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Incorporating expert knowledge in the Skin Allergy Risk Assessment (SARA) model: An integrated approach to testing and assessment (IATA) demonstrated in case studies

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There has been a successful effort to develop non-animal methods (NAMs) for skin allergy, combine these in defined approaches for hazard and categorical potency assessment and implement them in the context of flexible frameworks for next-generation risk assessment (NGRA). The SARA Model employs a Bayesian statistical model to define a human-relevant point of departure, the ED01, and the estimated risk associated with a consumer exposure by integrating the following data: the human repeat insult patch test (HRIPT), local lymph node assay (LLNA), direct peptide reactivity assay (DPRA), KeratinoSens™, h-CLAT, and/or U-SENS™. Here we share a significant development to the SARA Model which, through an expanded database, addition of further data sources and integration of expert information on chemical reactivity and sensitization potential, has allowed increased utility of the model as an integrated approach to testing and assessment.

The database was expanded from 81 to 428 chemicals. Additional data types, including historical published human maximization test and kinetic DPRA were added. Reactivity classifications were defined by expert chemists who reviewed database chemicals and the respective outputs from four *in silico* tools (Toxtree, OECD QSAR Toolbox, TIMES, and DEREK): Non-reactive, Non-reactive (autooxidation possible), Reactive, or Reactive (High Potency Category). These classifications bias estimates of the ED01 toward typical values associated with the assigned class. Low quantiles of these distributions were used to define exposure-based waiving (EBW) thresholds. *In vivo* data for database chemicals were reviewed, and expert-defined sensitizer/non-sensitizer classifications were assigned. A decision model was defined: to conclude low risk, the probability of low risk is > 0.95; to conclude high risk, probability of high risk is > 0.05; otherwise, the call is inconclusive.

Distributions of sensitizer potency for reactivity classes were reflective of published potency ranges. The updated model, using reactivity only, had improved overall classification performance (70%) against benchmark exposures when compared with published EBW thresholds (64%). Given all available data, the updated model correctly classified 83% of the risk benchmarks versus 64% with the previous version. The updated model was applied to the case studies previously described (doi:10.1016/j.yrtph.2022.105159), where it provided substantially higher confidence levels in the same conclusion versus the previous version. For example, using the same data, the previous lactic acid risk metric for a face cream exposure was 0.9, with the updated model this was 0.99 with a probability of non-sensitizer calculated to be 0.91 (91%). This version of the SARA Model is a significant improvement on the earlier version, allowing PoD and risk metrics to be defined whilst ensuring systematic inclusion of expert reactivity classifications in risk decision making.

Presentation: Oral



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Digital light processing 3D bioprinted artificial skin models

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3D artificial skin models provide versatile platforms for skin transplantation, disease mechanism studies, and biomaterial testing for skin tissues. However, creating complex physiological systems, such as the neurovascular network with living cells, within this stratified structure presents significant challenges. In this research, full-thickness skin models were developed using methacrylated silk fibroin (Silk-MA) and gelatin (Gel-MA), seeded with keratinocytes, fibroblasts, and vascular endothelial cells to represent the epidermis and dermis layers, utilizing a digital light processing (DLP) 3D printer. The printability, mechanical properties, and cell viability of skin hydrogels made from varying concentrations of Silk-MA and Gel-MA were analyzed to identify the optimal composition for 3D printing artificial skin. After fabricating the skin model with a bioink mixture of 15% Gel-MA and 5% Silk-MA, and culturing it for four weeks, histological analysis revealed healthy proliferation of keratinocytes and fibroblasts. Additionally, full-thickness skin wound models were 3D-printed to assess the wound-healing potential of the hydrogel. The DLP-printed artificial skin model exhibited mechanical stability and biocompatibility for over four weeks, indicating its potential for use in skin tissue engineering. This study successfully 3D-printed a full-thickness artificial skin model using silk fibroin and gelatin, which mimics the structural and cellular composition of human skin. The printed skin hydrogel supported cell viability and promoted healthy proliferation in both skin layers, as evidenced by histological analysis and immunofluorescence staining. The bioengineered hydrogel broadens the potential applications of artificial skin models for skin substitutes, wound healing studies, and drug testing.

Presentation: Oral

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Standardization and validation of cardiac organoids for safety and efficacy

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The increasing complexity of advanced biopharmaceuticals highlights the growing need for human-relevant *in vitro* models that can accurately predict cardiac responses. In this study, we successfully generated mature cardiac organoids from human pluripotent stem cells (hPSCs) and established standardized criteria for their quality evaluation and application in various safety and efficacy assessments. Beating cardiac organoids exhibited a consistent beating rate of 44.17 ± 3.61 BPM and showed robust expression of cardiac-specific markers cTnT and ML-C2v, confirming successful differentiation into functional cardiac tissues. Furthermore, a cryopreservation-thawing platform was applied, demonstrating that post-thaw cardiac organoids retained viability and functionality comparable to pre-cryopreservation levels, supporting the feasibility of long-term storage and large-scale production. We evaluated the electrical activity of cardiac organoids by monitoring Field Potential Duration (FPD) and QT interval changes. A 10% increase in FPD or QT interval (Yes/No) was used as a criterion to assess proficiency, reproducibility, and predictive accuracy. Additionally, amplitude changes were analyzed to evaluate electrical activity and contractile force. Functional alterations in beating frequency, contractile force, and calcium transients were observed following the treatment of various test compounds, highlighting the high sensitivity of cardiac organoids in detecting drug-induced cardiotoxicity. These findings demonstrate that cardiac organoids are a highly sensitive model for cardiotoxicity detection, overcoming the limitations of conventional animal models. This study provides a solid foundation for drug development, personalized medicine, and the development of alternative testing methods using cardiac organoids. Moreover, it aligns with international regulatory initiatives aimed at reducing animal testing, positioning cardiac organoids as a valuable model for preclinical research and safety assessment.

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Presentation: Oral



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VICH guideline on principles for technical guidance for the transition to *in vitro* methods for batch potency tests in veterinary vaccines

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The pharmaceutical industry still needs to use animals for developing and registering new veterinary medicines and for routine quality control, especially for vaccines.

With the application of the “3Rs” approach that aims to refine, reduce, and replace animal use, significant progress has been made in non-animal methods. This concerns, among others, veterinary vaccines, where some *in-vitro* release tests that have been developed demonstrated to be more reproducible, and cost-effective. They can indeed remove the inherent animal variability providing better discriminatory power compared to animal tests.

Not only do these methods allow to reduce the number of animals used, but by reducing the release lead-time and contributing to the batch consistency, the availability of products on the market is also secured.

However, two main hurdles remain:

1. Technical, as only few *in-vitro* release tests could be developed so far and
2. Regulatory: indeed, the differences in legislation across countries and lack of harmonization often lead the industry to run both *in-vivo* and *in-vitro* tests for the same batch, thus negating the benefits of these *in-vitro* tests.

This lack of harmonization perpetuates an unscientific and unethical situation and places a significant burden on multinational companies who want to develop and implement non animal methods. Developing a VICH guidance for transitioning to *in-vitro* methods has thus become crucial.

This talk will detail how the project of a VICH guideline on the transition to *in vitro* methods for batch potency tests in veterinary vaccines has emerged, the status of its development, progresses and the perspectives.

Presentation: Oral

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3Rs revolution: Transforming culture in large academic institutions

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Implementing the 3Rs – Replacement, Reduction, and Refinement at scale requires collaboration among scientists, leadership, key decision makers, and policy advocates. This presentation will use the University of Washington in Seattle, WA, USA (UW) as a case study to illustrate how embedding 3Rs principles fosters a culture of responsibility, innovation, and research excellence. Securing leadership and faculty engagement has been pivotal in embedding the 3Rs into research practices. By aligning initiatives with research excellence, funding competitiveness, and regulatory compliance, UW has integrated the 3Rs into planning, infrastructure, and education. Institutional assessments help identify gaps, set benchmarks, and measure progress.

Partnerships with leading 3Rs focused organizations such as the 3Rs Collaborative (3RsC), NC3Rs, and Norecopa have been key to UW's success. These collaborations provide access to research, best practices, and knowledge exchange, strengthening credibility, supporting standardization, and ensuring alignment with evolving global standards. At UW, cross disciplinary collaboration is essential for embedding the 3Rs across fields, including science that relies on animal models, micro physiological systems (MPS), artificial intelligence (AI), statisticians, clinicians, and data analysts. Researchers working on *in vitro* models, organ on a chip technology, and computational approaches collaborate to expand research tools. Statistical expertise and AI driven analysis enhance study design and refine model selection.

A strong 3Rs program requires scientific collaboration, training, and funding. UW has embedded 3Rs training into research education, new investigator onboarding, IACUC training, and continuing education for veterinary and animal care staff. Financial resources from the Office of Research have supported pilot programs and infrastructure improvements, identifying opportunities to improve research efficiency, reproducibility, and model development. As 3Rs discussions gain momentum at international, national, and state levels, organizations must align with evolving scientific and regulatory expectations. Engaging with policymakers, funding agencies, and government advocates ensures 3Rs principles are championed in institutional policies and broader research frameworks.

UW provides a scalable roadmap for fostering a 3Rs culture across disciplines, including science that relies on animal models, MPS, AI, policy development, and regulatory strategies. By addressing leadership engagement, funding barriers, and interdisciplinary collaboration, UW demonstrates how research organizations can implement lasting 3Rs initiatives. Attendees will gain practical insights into implementing a leadership supported, multidisciplinary approach to the 3Rs, ensuring their organizations remain at the forefront of responsible, high-quality research.

Presentation: Oral



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Glioblastoma organoids as patient's tumor avatars for *ex vivo* monitoring of treatment response to replace animal models in translational brain cancer research

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Glioblastoma is the most common and aggressive type of primary brain tumour associated with an abysmal prognosis. Clinical treatment for newly diagnosed glioblastoma patients has not changed in the last 15 years, revealing an urgent need to develop better preclinical tools that facilitate the rapid and efficient translation of new drugs to the clinic.

Preclinical animal models are used to test and evaluate the efficacy of drug therapies for glioblastoma. However, these animal models fail to accurately recapitulate human brain tumours' biology. Patient-derived glioblastoma organoids (GBOs) have recently emerged as a promising live animal-free model for studying glioblastoma. GBOs contain the human-relevant tumour microenvironment and accurately recapitulate the intratumor heterogeneity of tumour cells in each patient's tumour. Implementing patient-derived GBO models in the translational pipeline will lead to the development of functional precision medicine, which evaluates *ex vivo* therapy efficacy by treating GBOs in order to predict patients' responses within a clinically relevant time frame.

In this talk, we summarise recent key developments in standardising protocols for preparing the culture of GBOs, its advantages and disadvantages, and the key results of clinical trials using GBOs to guide personalised treatment of patients with glioblastoma.

Presentation: Oral

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Development of a human liver organoid-based platform for hepatotoxicity assessment

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The liver is the body's primary metabolic organ, responsible for the metabolism of substances, including pharmaceuticals, industrial chemicals, pesticides, and food additives. Some of these substances or their toxic metabolites can lead to acute liver failure or chronic liver diseases. Despite comprehensive non-clinical and clinical studies, drug safety issues continue to arise even after drug approval. Existing model systems lack the ability to fully recapitulate the complex structure and cellular composition of the human liver, limiting their usefulness in studying liver biology and assessing hepatotoxicity. Conventional *in vitro* human liver models, such as two-dimensional hepatic cells, lack *in vivo* physiological relevance, while animal models present challenges due to species differences and regulatory restriction. To address these limitations, three-dimensional human liver models, including organoids, are being actively developed. We have successfully established self-renewing and functionally mature human pluripotent stem cell-derived liver organoids and developed a hepatotoxicity evaluation platform. Notably, these liver organoids exhibited significant toxic responses to clinically relevant concentrations of drugs that were withdrawn from the market due to severe hepatotoxicity. Furthermore, the biochemical patterns of liver injury such as hepatocellular, cholestatic, or mixed-type damage were clearly distinguishable through liver function tests performed on the organoids, corresponding well with clinical data. In addition to drug toxicity testing, we are employing this platform to assess the hepatotoxicity of industrial chemicals and pesticides. The Korean Ministry of Food and Drug Safety is currently conducting validation studies on liver organoid-based toxicity evaluation methods in accordance with OECD GD34. A detailed review paper titled "Detailed review paper (DRP) on specific target organ toxicity (liver) test method using liver organoids, with a focus on human iPSC-based models for regulatory purposes" is being prepared in collaboration with the OECD Secretariat. We have also submitted a standardized document to ISO regarding the requirements and considerations for drug toxicity testing using human liver organoids. Taken together, our liver organoid-based hepatotoxicity evaluation platform holds great potential for various applications, including non-clinical drug toxicity evaluation before human trials, hazard identification, and risk assessment of substances that may impact human health.

Presentation: Oral



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Introduction to the role of the Korean Center for the Validation of Alternative Methods (KoCVAM) and standardization research initiatives for the practical application of new approach methods

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Korean Center for the Validation of Alternative Methods (KoCVAM) has been operating within the Ministry of Food and Drug Safety (MFDS) since 2009. As a member of the International Cooperation on Alternative Test Methods (ICATM), KoCVAM develops and validates new approach methods (NAMs) and strives for their international Standardization (e.g., OECD Test Guidelines (TGs), ISO, etc.).

KoCVAM has contributed to enhancing the competitiveness of the domestic industry by supporting the inclusion of NAMs developed and validated in Korea in the OECD TGs (TG442B, TG492 and TG439). In 2024, KoCVAM received OECD approval for two projects: one for developing a phototoxicity test using 3D models and another for preparing a Detailed Review Paper (DRP) in collaboration with the OECD on liver toxicity assessment using liver organoids. Also, KoCVAM has developed and validated a medical device skin irritation test method using human-reconstructed models for the biological safety evaluation of medical devices. This method has been proposed to ISO and is currently at the draft international standard (DIS) stage.

Since 2024, the National Institute of Food and Drug Safety Evaluation (NIFDS) has been conducting a five-years project “Standardization of alternative test methods for practical implementation”. The project aims to optimize and standardize NAMs (e.g., organoids, microphysiological system (MPS), and 3D tissues) for the safety, efficacy, and quality evaluation of pharmaceuticals and other substances, thereby enhancing scientific regulatory alignment and promoting their practical implementation.

Recently, the MFDS revised an administrative notice (Regulations on Pharmaceuticals Approval, Notification, and Review). According to the revised regulations, toxicity data included in drug approval review documents must be generated following Good Laboratory Practice (GLP). Among these data, non-animal or human biology-based models such as cell-based assays, MPS, bioprinting or computational models will also be recognized for regulatory acceptance.

The MFDS will establish a government-wide cooperative framework to develop and standardize NAMs, such as organoids and MPS. Additionally, in collaboration with the KoCVAM, the MFDS will continue to support efforts for the inclusion of these methods in OECD TGs and ISO Standards for medical devices.

This research was supported by a grant (25211MFDS002) from Ministry of Food and Drug Safety in 2025.

Presentation: Oral

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A roadmap to establish a framework leveraging advanced cell culture models for sustainable and locally relevant research in Africa

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The development of *in vitro* models such as induced pluripotent stem cell derived models and patient derived (disease) organoid models have highlighted the potential to develop population and patient-specific model systems reflecting human physiology and disease. However, currently non-European, and especially African model systems are greatly underdeveloped. This lack of relevant model development could seriously impact the investigation of population specific disease presentation, adverse effects and drug development. Unlike the genetic revolution of the late 90's and early 2000's lower to middle income countries, such as Sub-Saharan African countries, have a real opportunity to develop such models to direct their own research focused on the improvement of health of their own populations at a cost that is sustainable within the restricted funding environment they operate in.

To successfully implement such model development, national frameworks need to be implemented to direct funds and support such efforts in a way to maximize the output of advanced cell culture model systems while keeping the research at reasonable costs. To this end, a framework will be proposed, aiming to streamline the research efforts and simultaneously assuring the lowest possible costs. This framework ought to (i) consolidate and expand the capacity of infrastructure and human capital, (ii) initiate and facilitate the establishment of strong, long-term collaborations between national and international research groups, and (iii) provide secure, sustainable funding streams. The suggested framework includes three tiers, namely coordinators, generators, and collaborators. Briefly, the coordinators are funders or regulators on a national level identifying research areas with population-based disparities and launch competitive calls to develop appropriate model systems the generator tier can apply for. The generators themselves are national laboratories with expertise in model development that establish, annotate, validate, store, and distribute the model systems which can be used by the collaborators to investigate, e.g., disease mechanisms or treatment strategies. There is a strong research cooperation and knowledge transfer between generators and collaborators to apply the model systems and build more national expertise by training. Notably, generator laboratories can also serve as collaborators and utilize model systems for their research. The



aim is to build a living repository that provides model systems representing the diversity of the local population and therefore enables to investigate population-based health care disparities.

This framework truly presents an opportunity for low to middle income countries to base their healthcare research on locally relevant models to ensure that developed health care initiatives and interventions are best suited for the populations they serve and thus represent a reset in global health care research at large.

Presentation: Oral

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The GARDskin dose-response model to derive a PoD for quantitative skin sensitization assessment

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The regulatory acceptance of New Approach Methods (NAMs) for skin sensitization testing has significantly reduced the need for animal testing. The integration of these assays into OECD Test Guidelines (TGs) and Defined Approaches (DAs) enables hazard identification and semi-quantitative potency assessments aligned with the UN GHS system, with performance values comparable to or exceeding those of animal tests. While valuable for regulatory purposes, this potency information lacks the granularity required to conduct Quantitative Risk Assessments (QRA) or identifying safe concentrations of sensitizers in formulated products. Therefore, there is a need for assays that provide more quantitative, continuous potency predictions for use as Points-of-Departure (PoD) in risk assessments.

The GARDskin Dose-Response assay (GARDskin DR), is based on the GARDskin assay (OECD TG 442E) and incorporates dose-response measurements to derive potency predictions on a continuous scale. The readout in the assay is a cDVo value, representing the lowest concentration required to exceed the binary classification threshold in GARDskin. This value correlates well with skin sensitizing potency in humans and can be translated into a No Expected Sensitization Induction Level (NESIL) ($\mu\text{g}/\text{cm}^2$) using a simple linear regression model. Furthermore, the continuous potency predictions from the assay can be converted to UN GHS potency categories, by evaluating the continuous data with respect to a classification threshold of $500 \mu\text{g}/\text{cm}^2$ (LLNA EC3 of 2%).

The performance of GARDskin DR has been evaluated in blinded studies through collaborations between industry and method developers, with the findings summarized in a series of peer-reviewed publications. A meta-analysis of this data (> 200 chemicals) showed that the assay provides reproducible potency predictions ($n = 27$, 3 repeats), with average fold-change of 1.78 between repeats. The predictive performance was assessed using a subset of this data with highly curated reference data. Results indicated that the GARDskin DR predicted human NESIL values correlated well with human reference values ($r = 0.7$) and was a more potent predictor of skin sensitizing potency in humans compared

to the LLNA assay on the same dataset ($r = 0.6$). Furthermore, results showed that GARDskin DR was able to subcategorize test chemicals as cat. 1A or non-cat/1B with a predictive accuracy of 88.6% and 91.3%, when considering human or LLNA references, respectively.

This presentation describes the methodology, and the different datasets used to estimate performance of GARDskin DR. Two case studies will be presented to illustrate how the predicted NESIL value can be used in practice to ensure the safety of products. The first case study showcases how the assay has been integrated into a Next Generation Risk Assessment framework to identify a maximum allowable concentration of a sensitizer in consumer products. The second case study demonstrates how the assay can be applied during the development of new fragrance materials by identifying a safe concentration for confirmatory Human Repeat Insult Patch Testing.

In summary, this session introduces the GARDskin DR assay, present data to demonstrate the fit-for-purpose of this strategy, and highlights case studies on how the industry has already incorporated this approach into available frameworks for conducting QRA and to formulate safer product, without having to revert to animal testing, or to conduct human testing.

Presentation: Oral

**AGORA É LEI:
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From law to practice: How can EU Member States better protect animals in laboratories?

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Since 1986, the EU has had legislation on the protection of animals used for scientific purposes. The latest version of this legislation, adopted in 2010, led to Directive 2010/63/EU [O.J. L 276, p. 33- 79 (2010)]. This Directive sets standards for the accommodation, care, breeding, use and killing of animals, governed by the 3Rs principles. However, EU countries have faced difficulties in effectively implementing the Directive since its entry into force in 2013 (doi:10.1038/labani.604; doi:10.3390/biotech10020009; doi:10.1093/ilar/ilw029).

Inappropriate implementation of the legislation not only impacts animal welfare but also influences overall trends in animal uses. Under the EU Directive, Member States are required to collect and report annual statistics on the uses of animals for scientific purposes (doi:10.2779/595139). Although the EU Directive sets out “the final goal of full replacement of procedures on live animals for scientific and educational purposes as soon as it is scientifically possible to do so”, the reported uses of animals decreased by only 5% between 2018 and 2022.

The EU statistics indicate that policies to promote the development and use of non-animal technologies and approaches require a further push in order to significantly reduce the use of animals in science. Even after the development of non-animal methods to replace animal experiments for specific scientific purposes, it took decades to observe a decreasing trend in the number of animals traditionally used for these purposes. A striking example is the mouse ascites method, which continues to be authorized in some EU countries despite its near ban in most Member States, and the existence of valid non-animal alternative methods to replace it. Equally concerning is the upward trends in the use of some species, like the ones belonging to groups of non-human primates, especially in a context where human-based methods are thriving, and political commitments to phase out animals used in science are gaining momentum across the EU [O.J. C 290/01 (2023)].

In line with Article 54(1) of the Directive, Member States are required to submit to the European Commission information on its implementation once every 5 years. The analysis of the information submitted by EU Member States to the European Commission on the implementation of the Directive for the period 2018-2022 [SWD (2024) 183 final] enabled us to assess the effectiveness of the implementation of the Directive among 14 Member States that use the highest number of animals for scientific purposes. These Member States have improved some aspects of the implementation of the Directive compared to the period 2013-2017 [COM (2020) 15 final] intending to ameliorate the consistency and functioning of their national competent authorities. However, many of these authorities still miss, at some level, the expertise and competence

to identify areas where animals should not be used or should be replaced by non-animal methods.

The analysis also allowed us to develop a first set of recommendations to promote the adoption of best practices to ensure a harmonized, coherent and impactful implementation of the EU Directive across Member States. These recommendations can help to improve the protection of animals from their use in research, testing and education, and facilitate the achievement of the final goal of the EU to fully replace procedures on live animals for scientific and educational purposes.

Presentation: Oral

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The impact of barrier organ on bioavailable concentrations

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After exposure a xenobiotic enters the body and systemic circulation along the dermal, oral, or inhaled routes. Along the way it encounters barrier organs, the epitheliums of the skin, lung, or gastrointestinal tract. In the case of oral dosing the liver is also considered a barrier organ as it has a chance to metabolize the compound in question before it reaches the systemic circulation.

Bioavailability is defined as the fraction of an external dose that passes the barrier organs to reach the systemic circulation where it can have a pharmacological or toxicological effect.

This talk focuses on classical and advanced *in vitro* models for human absorption and metabolism, and how data from these experimental systems can be used in PBK-modeling to estimate systemic concentrations of xenobiotics in man to inform NAM-based toxicological models on realistic concentration ranges to be tested. Conversely, a concentration found to give a toxicological effect can be converted into an external dose.

In vitro models for the assessment of absorption, distribution, metabolism and excretion (ADME) properties range from cell lines, primary human cells and tissues to more advanced systems such as stem cell derived organoid cultures, for example intestinal organoids and liver spheroids and microphysiological systems. The advantages and limitations of these *in vitro* models for determination of ADME parameters will be discussed.

We have recently developed methods for the isolation of primary human jejunal enterocytes as well as 2D and 3D cultured intestinal organoids, enteroids, as well as liver spheroids. These *in vivo*-like *in vitro* models show promise to improve predictions of human absorption and metabolism of xenobiotics, especially as proteomic and functional investigations show a marked similarity of the models and human tissue.

Presentation: Oral



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Tools to deliver and achieve a culture of care at research institutions

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The promotion of a Culture of Care is essential for enhancing research quality, animal welfare, and human health. These are very closely related to each other. This presentation will explore different implementation tools aimed at fostering such a culture by taking starting point in our own work with Culture of Care. At Novo Nordisk, we started by making our own Culture of Care Survey, as a systematic approach to assess staff perceptions related to animal welfare. This empowered us to identify strengths and areas for improvement. This led to forming of a working group with the purpose of mapping the Culture of Care at all levels of the organization. Furthermore, we have given workshops and ensured targeted education focused on animal welfare best practices and ethical considerations, designed to deepen staff engagement and commitment to compassionate care. We ensure that there are feedback mechanisms in place to establish open communication channels that encourage staff to share their suggestions and concerns, promoting a responsive environment that values collective input. Furthermore, we have recognition programs, acknowledging and rewarding employees who exemplify the Culture of Care, thereby motivating staff to actively participate in enhancing animal welfare and ethical research practices.

By leveraging these tools, organizations can create a supportive and ethically driven research landscape that not only prioritizes animal welfare but also fosters innovation and collaboration. This presentation will illustrate how actively working with cultivating a Culture of Care contributes to improved research outcomes, ensures ethical integrity, and ultimately benefits all stakeholders involved in the research process.

Presentation: Oral

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NAMs: Perspectives and challenges in supporting innovation in industry

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Historically, chemical industry and chemical regulations have relied on animal testing as a fundamental method for conducting chemical safety assessments. Despite notable progress in replacing animal studies in particular in the field of local toxicity, acceptance of alternative methods remains limited. The chemical industry is investing in new approach methodology (NAM), including computational toxicological models, grouping, and *in vitro* assays, to evaluate hazard of chemicals and risk from their exposure. At the same time a modernized regulatory framework is needed that leverages scientific advancements, enabling benefits for public health and economic outcomes while ensuring safety, technological feasibility, and predictability for industry stakeholders.

Today the options to fulfill mandatory information requirements without animal testing are limited. To ensure the effective and targeted assessment of chemicals, legislation must be modified to incorporate NAMs (here non-animal methods) alongside potency and exposure considerations.

NAMs can be utilized for establishing safe levels of exposure (points of departure, PoD). These NAM-derived PoDs are frequently more conservative than traditional PoDs. Yet, further effort is necessary to incorporate meaningful exposure considerations and minimize uncertainty. Moreover, trusted, and usable data require standardized methods which have been developed with assured quality (OECD good *in vitro* method practice, GIVIMP) and were meticulously validated for relevance and reliability – and are then used in standardized and quality-controlled procedures (GIVIMP and good laboratory practice, GLP). Such NAMs can provide reliable information on human-relevant bioactivity, potency and bioavailability of chemicals, and guide decisions regarding the necessity for higher-tier testing. However, caution is warranted when interpreting *in vitro* results derived from high concentrations, as their relevance may be limited. It is crucial to establish and reach consensus on workflows for the appropriate interpretation of such data – such as determining meaningful concentrations for *in vitro*.

To ensure competitiveness and predictable timelines, NAM methods mandated by regulations must be standardized and provided by multiple certified labs. To avoid contradictory results, data shall be generated from standardized NAMs which are universally accepted across regions and regulations. Achieving this necessitates internationally harmonized application of NAMs and mutual acceptance of data (MAD). Industry's collaboration and dialogue with regulators and the scientific community are vital for building trust and the exchange of post-validation findings regarding the use of robust NAMs.

At the same time, industry is developing and using NAMs to guide their product innovation. This use of NAMs is often targeted towards a specific group of chemicals and their technological application. Establishing the readiness or qualification of NAMs for such applications is different from regulatory applications, because of their targeted application, their fit to the development target and the innovation cycle. Here, NAMs are essential to implement safety considerations into product development as a major pillar of safety and sustainability by design (SSbD). Obviously, both applications of NAMs can advise each other and may converge as requirements in innovation reflect regulatory demands – and *vice versa*.

Presentation: Oral



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Bringing preregistration to 3Rs education: A new ETPLAS E-module

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Context: Preregistration, the act of publicly registering a detailed research protocol before starting a scientific study, is a mandatory step for clinical trials, and it has recently gained traction in animal-based biomedical research, with the launch of two dedicated platforms for preregistering of animal studies. Given its benefits to the transparency, integrity, and reproducibility of research, it is expected that similar platforms will soon be available for *in vitro* research.

Preregistering research protocols helps improve accountability and transparency by sharing in advance the hypotheses, study design, and analysis plan. Moreover, it serves reproducibility and integrity by describing details that might go missing in manuscripts, and by reducing the risk of reporting biases, and questionable research practices such as p-hacking and HARKing (Hypothesizing After Results are Known). It also furthers the 3Rs principle of Reduction by contributing to avoid involuntary duplication of studies and by making all studies available, including interrupted or unpublished ones.

Issue: Despite its benefits, the adoption of preregistration remains limited in biomedical research. Barriers include lack of awareness, misconceptions about the method (e.g., perceived administrative burden, concerns about scooping and lack of scientific creativity), and challenges in integrating new topics into already packed curricula. As a result, preregistration is not incorporated into 3Rs education globally, limiting its potential to improve research quality and reduce unnecessary animal use.

E-module content: To address this gap, Preclinicaltrials.eu and the Education and Training Platform for Laboratory Animal Science (ETPLAS) have developed an interactive e-learning module on preregistration for biomedical research, supported by a grant from Laboratory Animals Limited. The module covers the principles, benefits, and practical applications of preregistration of animal studies, also generally applicable to non-animal studies. Learning is supported through text, images, interactive tools, and videos, with optional reading materials for further exploration. This short, interactive e-learning module is well-fitted for researchers, students, and any stakeholders wishing to learn about preregistration. Its flexible format allows for easy integration into existing courses on research integrity, open science methods, research transparency or mandatory laboratory animal science training, as well as for undergraduate and post-graduate education. The official launch is planned for Spring 2025.

Future implementation: We aim to integrate this module into educational programs worldwide to create broader awareness and adoption of preregistration. We will utilize user feedback to guide refinement, ensure relevance across disciplines and maximize its impact on research. As research on preregistration continues, we will keep references and examples up-to-date with ongoing literature.

Presentation: Oral

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Advancing alternative methods in Argentina: The role of LMA in promoting non-animal toxicity testing

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The Laboratory of Alternative Methods (LMA-EBAL) is a pioneering institution in Argentina, promoting the implementation, development, and dissemination of alternative methods for toxicological evaluations. Since its establishment in 2016, LMA has focused on incorporating internationally validated methodologies, developing new *in vitro* strategies, and fostering collaboration between academia, industry, and regulatory agencies to accelerate the acceptance of non-animal approaches in Argentina and Latin America.

LMA's initiatives include organizing scientific events, training workshops, and regulatory discussions to enhance knowledge transfer and promote adoption of alternative methodologies. Key activities include hosting the pre-congress course for COLAMA 2018, coordinating the annual MAPEF workshop since 2019, and leading regional events with MERCOSUR, such as PreMASUL 2021. Additionally, hands-on training programs like the Episkin Training in 2022 and the *In vitro* Toxicology Training Workshop in 2024 have provided practical experience in alternative approaches. LMA has also worked with companies to implement *in vitro* assays for ocular and skin irritation, corrosion, and sensitization, leading to their adoption by local industries.

Despite regulatory challenges, LMA's work has positioned alternative methods as a viable and necessary approach for safety evaluations in Argentina. This presentation will provide an update on global, regional, and local advancements in alternative methodologies, highlight LMA's role in replacing animal testing, and discuss strategies to further integrate non-animal models into regulatory frameworks. By sharing these developments, we aim to catalyze innovation and strengthen the adoption of the 3Rs principles across Latin America.

Presentation: Oral



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The EU Partnership for the Assessment of Risks from Chemicals

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New approach methods (NAMs) have the potential to become a major tool for regulatory risk assessment; however, the actual implementation is challenging. The European Partnership for the Assessment of Risks from Chemicals (PARC) has been designed to address various challenges associated with innovating chemical risk assessment. PARC's proximity with national and European regulatory agencies aims to ensure that the research and innovation projects initiated within PARC coincide with actual regulatory needs.

One of the challenges is related to validating NAM for regulatory use. While full formal validation is not a task of PARC several aspects of validation are addressed. This includes co-design of methods by taking into account the regulators perspective in an annual peer review of all projects as well as evaluation of readiness of methods and transferability assessment for advanced methods.

Another challenge is related to how to come to regulatory conclusions based on NAM can be drawn.

A third challenge is related to the fact that systemic toxicity studies in animals normally address numerous potential hazardous effects in multiple organs at the same time. By focusing on adverse outcome pathways (AOP) and combining several NAM to endpoint specific IATA-based test batteries PARC addresses this for the endpoints non-genotoxic carcinogenicity, metabolism disruption, endocrine disruption, immunotoxicity and developmental (neuro-) toxicity.

The present talk will give an overview on the challenges listed above and how PARC is addressing them.

Presentation: Oral

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Opportunities to build confidence in use of NAMs for systemic toxicity

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Many environmental chemicals are data poor and lack traditional toxicological datasets that are typically used for conventional identification of toxicity thresholds. In this context, alternative methods can be used to augment chemical evidence by filling data gaps and reducing uncertainties with the existing information. EPA's Office of Research and Development (ORD) produces high quality and scientifically rigorous chemical assessments to inform decisions made to protect human health and the environment as mandated by a variety of statutes. Federal agencies address the challenges of environmental exposures and contamination by ensuring science-based decision-making, which can include

filling gaps in understanding of the potential human or ecological health hazards associated with chemical exposures. New approach methods (NAMs) have the potential to advance scientific understanding in support of timely human and ecological health risk assessments. In support of the strategies outlined in the Next Generation Blueprint of Computational Toxicology at the US EPA (2019) and the EPA NAMs Work Plan (2021), collaborative research efforts have been ongoing toward development of novel methods that support assessment of point-of-departure and that are contextualized with exposure information on the chemical. This talk will detail some of the advances in NAMs under development at the Agency – and in collaboration with partners – that may inform updates to health assessment strategies. In addition, efforts to develop frameworks for validating and building confidence in NAMs, both nationally and internationally, via the Interagency Coordinating Committee for Validation of Alternative Methods and the Organisation for Economic Cooperation and Development, will be discussed. Taken together, research and development efforts grounded by international standards for validation and strategic partnerships will be crucial to the development of scientifically robust and validated NGRA frameworks.

The views expressed in this abstract are those of the author and do not necessarily represent the views or policies of the U.S. EPA.

Presentation: Oral

Six decades leading the science supporting fragrance safety

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From animal testing to regulatory approval: Evidence-based approaches in drug discovery

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Background: Animal experiments are often justified by their potential to benefit human health. However, the extent to which findings from animal studies translate to humans remains debated, particularly in drug development. Systematic reviews, which provide structured summaries of available evidence using explicit methods, help maintain an evidence-based discussion despite the vast volume of published research. They also play an important role in informing the 3R principles.

Objective: Our presentation will demonstrate four ways systematic reviews reveal systemic issues in animal research and support translation.

Results: First, an umbrella review of 367 therapies tested in animal models across 51 diseases found that only 5% of these therapies eventually received regulatory approval for human use.

Second, we examined how often animal testing continues after regulatory approval by analyzing publication data from PubMed and Embase for 41 drugs approved by the FDA between 2011 and 2014. More than 70% of animal studies conducted post-approval focused on drug testing, with a significant portion assessing efficacy despite the drug already being approved.

Third, a large systematic review extracting data from 1243 animal studies on psychedelics such as LSD, psilocybin, and DMT found that the applied human-equivalent doses in animals were, on average, six times higher than those used in clinical studies.

Fourth, a systematic review assessed the translational potential of Salvinorin A, a kappa opioid receptor agonist, for psychiatric applications.

Discussion and conclusion: Systematic reviews expose systemic challenges in animal research, highlighting issues relevant to both the 3R principles and human health. They also improve the understanding of translational pathways from animal studies to human applications. We call to action for more and rigorous systematic reviews among animal researchers to ensure researchers base their decisions on the best available evidence. We also advocate for strengthening collaboration between animal and human researchers to reduce research waste and improve translation.

Presentation: Oral

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The path to better science: Practical advice on implementing the 3Rs and related principles in animal research

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Great progress has been made in developing means of reducing and refining animal use in science. Scientists must be given easy access to these developments, and this presentation will showcase how this is being done. On the other hand, the third R of Replacement of animals in research and testing (particularly in academic circles) is not advancing at the same rate, as it involves more of a paradigm shift. Many non-animal methods that are under development do not have the primary aim of replacing *in vivo* experiments, and the work is often carried out by scientists who do not have contact with animal facilities. This can lead to an inherent scepticism to replacing a long-standing animal model with a new protocol with which they have no experience, and which will need to be validated.

The path to better science involves, therefore, close collaboration between experts in a variety of fields, including laboratory animal science and facility management, experimental design, statistical analysis and a variety of non-animal methods.

Collaboration stems from good communication, bringing 3R advances to the table in a spirit of cooperation, for the ultimate benefit of science and animal welfare. It is particularly important that the two sides – proponents and opponents of animal use – contribute constructively to this discussion, without over-selling the methods they believe to be best. For many scientists using animal models, the new world of New Approach Methodologies appears to lie far from the needs they feel to study a complete biological system.

The flood of information about 3R advances needs to be channeled into outlets which scientists can easily relate to within a busy schedule. For many years, efforts to improve science using animals focused more on better reporting than on better planning and evaluation of non-animal methods. This imbalance has been corrected by the development of guidelines to help scientists consider all three Rs from day 1 of planning, even if they had intended to use a familiar, validated animal model.

This presentation will showcase the work being done to help researchers make the right decisions on the path to better science, whether that path involves animals or not.

Presentation: Oral



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A call to action: Advancing new approach methodologies (NAMs) in regulatory toxicology through a unified framework for validation and acceptance

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Ensuring the safety of chemicals and pharmaceuticals while minimizing reliance on animal testing is a 21st century toxicology challenge. Traditional animal models have limitations in predicting human responses and raise ethical concerns. Development and adoption of New Approach Methods (NAMs) has resulted. NAMs, encompassing *in vitro*, *in silico*, and *in chemico* techniques, offer the potential for more human-relevant, and ethically sound, safety evaluations.

A lack of standardized validation and acceptance criteria is a challenge. NAMs data has improved reliability and relevance for predicting human toxicity compared to traditional animal tests. Successful case studies of NAMs implementation, regulatory perspectives and initiatives are evaluated. This analysis reveals a need for a unified, cross-industry approach to NAMs validation, grounded in measurable quality standards and standardization. A framework is proposed based on clearly defined standards, standardized protocols, and transparent data sharing to accelerate the integration of NAMs into regulatory decision-making, ultimately benefiting human health, animal welfare, and scientific progress.

These recommendations have been proposed.

1. Establish a Global NAMs Initiative representing stakeholders involved in test system and method development, implementation, validation coordination, and development of best practices.

2. Develop a Centralized NAMs Knowledge Base containing available NAMs technologies, identifying those ready for implementation or requiring further development. A curated Reference Chemical Database with well-characterized toxicity profiles should be made available for use in validation studies. Data should be openly available from clinical trials as well as traditional *in vivo* tests.
3. Prioritize NAMs Validation and Address Funding Bias. Advocate for funding mechanisms that prioritize independent validation studies, shifting resources away from focusing on new test development which require validation for acceptance. Establish clear criteria for prioritizing NAMs for validation based on scientific need, regulatory impact, and feasibility.
4. Develop, and Promote Education and Training Resources. These should be created to educate all stakeholders on NAMs principles, applications, validation procedures and interpretation of results backed up with comprehensive training programs.
5. Foster International Harmonization. Encouraging data submission using NAMs data. Work with all regulatory agencies to develop clear guidelines for the acceptance of NAMs data in regulatory submissions.
6. Specific Stakeholder Engagement. Test method and test system developers should collaborate with the initiative to define validation study parameters, share data, and participate in interlaboratory studies. CROs should promote involvement in validation studies according to standardized protocols. Regulatory Agencies should encourage early and frequent dialogue with NAMs developers and provide guidance on validation requirements and acceptance criteria. When NAMs data is submitted, provide clear feedback on how the NAM has been received, and if it has been used in the decision-making process.

All stakeholders are urged to actively participate in this initiative by contributing their expertise, sharing data and insights, and collaborating on the development and implementation of this crucial use of NAMs in regulatory toxicology.

Presentation: Oral



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Validation powerlifting: Strengthening confidence in NAMs with flexibility and fitness (for purpose)

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New approach methodologies (NAMs) are increasingly being used to assess potential toxic effects of chemicals and products on human health. However, as modern approaches to toxicity testing have evolved (e.g., from replacing *in vivo* tests with a single alternative assay to the more contemporary practice of integrating results from multiple alternative approaches), so has the need to adapt the assessments of a NAM's scientific validity. This presentation will summarize how this need is being addressed by recent activities of the U.S. federal Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).

As described in the ICCVAM 2024 report, "Validation, Qualification, and Regulatory Acceptance of New Approach Methodologies," confidence in a NAM should not be based entirely on historical approaches such as the outcome of a ring-trial study or exclusive comparison to reference animal data. Instead, confidence should be strengthened through flexible, fit-for-purpose validation strategies that consider a NAM's intended application. Key concepts include consideration of the NAM's context of use, biological relevance to the species of interest, technical characterization, data integrity, information transparency, and independent review.

The report emphasizes that establishing confidence in NAMs is an iterative process that requires communication among method developers, regulators, and validation bodies. NAMs developers need to understand end-user needs to assure that the method is fit-for-purpose (i.e., provides information that is scientifically sound and meets requirements for the intended regulatory decision or use case). When appropriate and feasible, stakeholders should communicate if or when a NAM is acceptable for their needs.

To implement the report's concepts, ICCVAM is organizing Method Developers Forums (MDFs), each iteration focused on a specific toxicity/endpoint. To prepare for the MDF main event, NAMs developers review materials summarizing stakeholder information requirements and/or decision frameworks relevant to the topic. Each proposal submission describes their method and how the key concepts have been addressed

in validation efforts. Developers are then invited to present their method and how it may be useful for stakeholders at the MDF main event. A panel of stakeholders provides feedback on the featured NAMs' readiness, confidence, and other aspects that should be addressed to strengthen the validation efforts. Thus, the MDF provides an opportunity for reciprocal communication among NAMs developers and intended end-users that can help advance NAMs toward implementation and regulatory acceptance.

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Presentation: Oral

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Assessing data integrity: A deep dive into data review in method validation

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Data review plays a pivotal role in establishing the credibility and reliability of experimental outcomes during scientific method validation assessment. This presentation aims to dissect the data review process within the context of a scientific method validation packet. Key components of the data review process used to assess the accuracy, completeness, and integrity of the submitted data are discussed. The test submitter's data is traced, from its capture as raw data on the laboratory equipment, through its presentation in a validation packet. Tools to help in this process, like data flow diagrams and features within data analysis software like audit trails and metadata capture are covered. Real-world case studies are presented, offering insights into common challenges encountered during the data review phase and the strategies employed to address them, where possible. Emphasizing the importance of data governance strategies, especially in the context of multi-laboratory endeavors, the presentation underscores their role in safeguarding the integrity of both present and future laboratory and validation data. These concepts, though derived from Good Laboratory Practices and Good *In vitro* Method Practices, extend their relevance to any scientific work environment. Solid data integrity is necessary in assessing a method's validity and a robust data review process is essential to support data integrity.

Presentation: Oral



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Courses in study design: Overview and experiences

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Designing robust, reproducible animal studies is both a scientific and an ethical imperative. While good experimental design is often mainly placed within Reduction, it must consider all 3Rs. Indeed, the harm-benefit analysis on which the ethical appraisal of animal studies is based aims at answering the question: “is it worth using sentient animals?”. Hence, study design must first consider Replacement and whether the scientific study with animal is justified in terms of non-animal methods available, animal numbers, potential suffering, potential benefits, and likelihood of achieving them.

Refinement is also at the core of study design, through the choice of techniques, instruments, anaesthetics/analgesics, housing, husbandry, handling, palliative care, and how early in disease progression will scientific objectives be obtained, among other factors. Such decisions should not only aim to prevent or mitigate aversive sensations like pain or distress, but also more nuanced ones such as boredom and loneliness, aiming to provide enough positive experiences as to grant laboratory animals, if not a good life, at least a life worth living.

In order to be scientifically and ethically justified, animal studies should not only uphold high methodological standards but also fully consider all 3Rs principles, in the planning, execution, analysis, and reporting stages. The EU lists several 3Rs-related learning outcomes for those receiving training to design procedures and projects, i.e., EU-function (b). However, no experimental design training is mandatory in Europe for those carrying out procedures in animals (which only requires EU-function (a)). Given how intricately connected study design choices are to the 3Rs, I will argue against allowing anyone to carry out procedures in animals without prior education and training in experimental design. I will stress how those performing interventions susceptible to cause animals pain or distress should know not only how to perform them, but also whether to do so, when faced with a particular scientific rationale, methodological choices, available technical expertise, logistic constraints, and available alternatives. This would also not only equip those using animals with much needed knowledge to identify flawed designs but also give them the agency and authority to challenge those proposing them, thus avoiding wasting animal lives, time and resources.

Dedicated training in Experimental Design, that includes animal welfare and the 3Rs, is an opportunity to not only raise awareness about fundamental methodological standards (such as randomization, blinding, adequate statistical power and proper experimental units) but also to discuss the scientific and ethical consequences of getting it wrong. This talk will provide an overview of such training efforts in Europe, share experiences from organizing and delivering courses, and describe the contributions of the FELASA working group for experimental design education.

Presentation: Oral

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Recent research trends in advanced alternative testing methods using lung organoids

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This talk will cover the evaluation method for acute inhalation toxicity using lung organoids, a field that has been actively researched in recent years. Specifically, it will discuss research trends in lung organoid-based experimental methods as an alternative to conventional animal testing for acute inhalation toxicity assessment. Additionally, the presentation will explain experimental data on acute inhalation toxicity evaluation using lung organoids and aim to confirm their potential as a replacement for animal experiments.

In our experiment, we used airway organoids (AOs) derived from primary human airway epithelial cells (hAECs) for AO-based acute inhalation toxicity screening. We proposed a pillar platform (pillar-dish, 384-pillar/well plate) for culturing airway organoids. A 20 μ L mixture of hAECs and Matrigel (50% v/v) was dispensed onto a 5 mm diameter pillar surface in a pillar plate, forming a uniform dome of hAECs/Matrigel. To confirm AO formation, H&E staining and immunohistochemical (IHC) analysis were performed using anti-p63 (for basal cells), anti-MUC5AC (for goblet cells), and anti-FOXP1 (for ciliated cells) antibodies. As a result, we demonstrated that human AOs cultured on the pillar surface exhibited the histological characteristics of hAECs.

For toxicity models, a 3 μ L mixture of AOs and Matrigel (50% v/v) was re-dispensed onto a 2 mm diameter pillar surface in a 384-pillar/well plate. Toxicity screening was conducted by treating AOs with toxicity-positive and negative compounds in the 384-pillar/well plate and analyzing them using cytokine and ATP assays. The toxicity-positive and negative compounds were classified according to TG436, the guideline for animal testing on the maximum concentration for acute inhalation toxicity. The positive compounds included: 2-Butoxyethanol, Pyrrolidine, 2-Methyl-4-isothiazolin-3-one, and Thioglycolic acid. The negative compounds included: 1,3,5-Trioxane, 1,3-Dioxolane, tert-butyl methyl ether, Acetone, Ethyl alcohol, Methyl methacrylate, 1-Propanol, Dimethyl sulfide, and Glycerol.

In the AO-based toxicity test, toxicity-positive compounds exhibited higher acute inhalation toxicity (IC₅₀ lower than 20 mM) compared to negative compounds (IC₅₀ higher than 100 mM). Therefore, the proposed AO-based toxicity test can be effectively used as tools for acute inhalation toxicity screening.

This research was supported by a grant (RS-2024-00332142) from Ministry of Food and Drug Safety.

Presentation: Oral



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Young TPI: Empowering the next scientific generation to go animal testing-free

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The Transitie Proefdiervrije Innovatie (TPI) initiative, a collaboration between the Dutch government and organizations involved in animal(-free) research, was launched in 2018 to shift the focus from reducing animal studies to accelerating animal-free innovations. Recognizing that this transition may take one or two generations, the initiative actively involves the next generation of young professionals and students. In response, Young TPI was created as a dynamic network to empower young people in the field of animal-free research.

Young TPI serves as a platform for engaging young people in ongoing initiatives to make animal testing free. The network enables young scientists to connect and collaborate across diverse research areas, from fundamental to translational and regulatory research, to advance the acceptance and implementation of animal-free methodologies. It achieves its goals through three core pillars: stimulating the transition by encouraging collaboration with ambassadors and creating incentives for change, raising awareness and showcasing the possibilities of animal-free alternatives through lectures and courses, and stimulating a network for knowledge-sharing and experience exchange.

Since its inception, Young TPI has grown a large network across the Netherlands, attracting over 300 members from different institutions, including biotechnology startups, pharmaceutical companies, and universities. The network's diverse membership provides valuable expertise in advancing animal-free research. With a vision to extend its influence across Europe, Young TPI is committed to empowering the next generation of scientists to drive ethical, innovative, and accurate research methodologies that prioritize animal welfare and enhance scientific progress with animal-free innovations.

Presentation: Oral

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Integration of metabolism into ASPA – A framework to implement NAM-based approaches into regulatory risk assessment

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Chemical compounds can undergo metabolism to form metabolites that may exhibit toxicity levels and/or effects different from their parent compounds. In the evaluation of such compounds using New Approach Methodologies (NAMs), a structured approach is needed to efficiently prioritize metabolites for *in vitro* testing. In the Horizon2020 EU project RISKHUNT3R, we have developed the Alternative Safety Profiling Algorithm (ASPA), a framework for next generation risk-assessment of compounds using NAMs. The conazole case study (CS) in RH3R aims to develop a next-generation risk assessment strategy that not only predicts the hazard potential of parent compounds but also characterizes the formation and toxicity of their metabolites using NAMs. Conazoles can metabolize into active metabolites, including hydroxy derivatives and 1,2,4-triazole. *In vivo* toxicity data suggests that conazoles and 1,2,4-triazole cause distinct toxicities. To address this, we utilized *in silico* and *in vitro* tools within the RH3R toolbox to assess the toxicity of parent conazoles, their hydroxy metabolites, and 1,2,4-triazole. These findings contributed to the development of ASPA for assessing compounds with active metabolites, enhancing the efficiency and predictive power of risk assessment strategies.

Presentation: Oral



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The role of integrated assessments and regulatory challenges in environmental safety assessment

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Rising environmental concerns and new regulations are driving the need for improved environmental safety assessments. L'Oréal has been involved for years in the development of OECD test guidelines using New Assessment Methods (NAMs) for endpoints like bioaccumulation, acute aquatic toxicity, and first level of endocrine disruption assessment, but gaps remain. Further development is needed in calibrating bioaccumulation models, creating *in vitro* and *in silico* methods for chronic fish toxicity, addressing the WHO endocrine disruption definition for vertebrates without *in vivo* tests, and developing alternative methods predictive of effects on vertebrate progeny. Many industrial sectors are working within EPAA (European Partnership for Alternative Approaches to Animal Testing) on recommendations to the European Commission for actions regarding the acceptance of alternative methods to address environmental safety and biodiversity protection. Industry initiatives and collaborations, such as ICCS (International Collaboration on Cosmetics Safety) for cosmetics ingredients, are establishing a scientific roadmap for environmental alternative methods. Continuous development and collaboration are crucial for assessing the environmental impact of cosmetic ingredients while adhering to animal-free testing and ensuring product sustainability.

Presentation: Oral

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Mission impossible accomplished? The harm-benefit analysis between policy, practice, and progress

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The regulation of animal use in scientific research is governed by specific laws in many countries, with Directive 2010/63/EU (the Directive) serving as the primary legal framework in the European Union (EU). While the Directive aims to harmonize legislation among EU Member States (MS), its provisions allow for flexibility in interpretation and give MS room to maneuver, which may lead to discrepancies in national legislation and inconsistencies in its implementation. One key aspect affected by this variability is the project evaluation process, particularly the harm-benefit analysis (HBA), which serves as an essential tool for justifying the use of and harm inflicted on animals.

Despite the Directive's intent to establish harmonization among MS, the challenging operationalization of the HBA has been debated at length and remains incomplete. Our study examined the transposition of the Directive's HBA requirement into national legislation across EU MS, identified relevant national and regional guidance documents, and conducted a comparative, cross-national analysis of the HBA's implementation. The results, previously shared in other discussions, highlight significant deficits in the transposition of the HBA requirement, substantial inconsistencies in available guidance documents, and a lack of coherent implementation. These findings underscore the need for a structured HBA approach to ensure transparency and consistency in the evaluation and decision-making process by reviewing committees.

To address these challenges, future approaches to HBA may benefit from integrating key components of the existing HBA guidance documents, merging them into an adaptable and practical tool. This presentation will include a brief overview of HBA concepts, an update on the status quo of HBA implementation in EU countries, and an outlook on how a new HBA methodology may advance current frameworks and promote greater consistency across reviewing processes.

Presentation: Oral

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Ready for prime time: Readiness criteria for supporting validation

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The concept of Readiness Criteria (RC) for evaluating the readiness of methods for validation and regulatory acceptance was first introduced by Bal-Price et al. in 2018 [1], based on OECD Guidance Document 211 for describing non-guideline *in vitro* test methods [2]. Since then, various RC templates have been developed, each with similarities and differences in criteria and purpose [3-7]. The availability of multiple resources and the lack of international agreement on a standardized approach pose a challenge for method developers and regulatory authorities. To address this, the Project Group (PG) on the revision of the OECD Guidance Document (GD) 34, co-led by the European Commission Joint Research Centre (JRC), the US, and the Netherlands, aimed to develop harmonized templates for evaluating the readiness of methods and approaches at different stages of the validation workflow. The ultimate goal is to facilitate the development and acceptance of OECD Test Guidelines.

The JRC developed a template for individual *in vitro*, *in chemico* and *ex vivo* test methods, designed to be fit-for-purpose for the OECD Test Guidelines Programme (TGP). This template was informed by existing resources, including the ECVAM Test Submission Template and the OECD Guidance on Good *In vitro* Method Practices (GIVIMP).

The template is endpoint-agnostic and covers five key categories: (i) test method definition and description, (ii) data management, evaluation, and interpretation, (iii) test method reliability, (iv) test method relevance, and (v) data integrity and quality assurance. It is intended as a self-assessment tool for method developers and a prioritization/evaluation tool for regulatory authorities, such as validation bodies, OECD, and financing bodies.

The template only includes essential criteria, and no scoring system is proposed. Instead, it defines the essentiality of each criterion for a method to enter validation, to be transferred to a naïve laboratory, or to be peer reviewed. While all criteria are essential for peer review, they may not be necessary at earlier stages of the validation workflow. The proposed template provides a comprehensive framework for adequate method development and for evaluating whether a method is mature enough to be validated and peer-reviewed for inclusion in the TGP.

Building on this work, the Project Leads proposed developing separate templates for individual *in silico* methods, Defined Approaches, and *in vivo* methods, with the US offering to take the lead on this effort. The inclusion of internationally agreed RC in the revised OECD GD 34 will be a valuable resource for those involved in the development, validation, and acceptance of methods for hazard and risk assessment.

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Presentation: Oral



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Safe and sustainable by design workflows supporting product design and decision making

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Traditional product safety assessment processes frequently rely on lengthy animal testing and may fail to accurately predict and manage impacts during product design and innovation on human and environmental health and their interactions, highlighting the urgent need for a move toward more effective and timely assessment methods.

Within our framework, New Approach Methods (NAMs), such as high-throughput screening, *in silico* and *in vitro* methods, and omics technologies, are integrated into tiered strategies involving Safe and Sustainable by Design (SSbD), Integrated Approaches to Testing and Assessment, and Next Generation Risk Assessment, to incorporate function, safety and sustainability into product design from the start of the innovation and development process.

Our developed methodology is founded on knowledge integration of data, models and supporting evidence incorporated into a workflow system implementing and documenting SSbD tasks and decisions with trusted integrity and reproducibility.

We will explain how a tiered iterative workflow approach enables users to use SSbD methods and tools effectively, hence improving their ability to integrate the concepts and calculations into daily operations and product decisions. Through application to a variety of industrial case studies, we demonstrate how this approach works in real-world scenarios, guiding early product design decisions ensuring that products are optimized for function, safety and human and environmental health impacts.

We discuss the use of modelling and AI techniques to assist the collection, generation and use of evidence as a critical strategic NAM methodology supporting SSbD goals. Valuable assistance is obtained from resources that use data and scientific techniques to obtain and organize relevant knowledge embedded into SSbD decision-making workflows. To achieve this goal, full use of existing data, models and knowledge should be leveraged into providing relevant information supporting assessment and decision goals between alternatives in early-stage innovation. Such data needs to be integrated with evidence-weighting and scoring schemes, including uncertainty, to reach initial decisions on alternatives and to plan for subsequent refinement phases. The findings may include recommendations for generating the most meaningful and useful data to address gaps and uncertainty. We will demonstrate the methodology followed and questions arising from current case study work applying SSbD to chemicals, polymers and advanced materials.

Presentation: Oral

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AI-assisted next generation risk assessment and safe and sustainable by design workflows enabled by FAIR data and knowledge

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We describe our work in preparing datasets, models and knowledge graphs according to FAIR (Findable, Accessible, Interoperable, Reusable) principles connecting compound information, biological response data, pathways and key events based on our experiences in developing knowledge resources supporting Next Generation Risk Assessment (NGRA) and Safe and Sustainable by Design (SSbD) case studies, applications and workflows. Example knowledge resources to be discussed include:

- FAIR datasets, models and knowledge graphs supporting New Approach Methods in Toxicology;
- integration of knowledge in workflows supported tiered testing and assessment strategies for NGRA problem formulations and case studies;
- integration of evidence for the assessment of ingredients and formulations using tiered SSbD workflows.

The related open knowledge resources include harmonized data templates for organizing research data according to FAIR principles; omics data management best practices and implementation; approach to collected data including harmonization, referencing and integrity goals; compound data management including approach to compound identifiers, properties, project and case study annotation, characterization and linked biological and toxicological data, biological models and support for user-oriented hypothesis testing.

Such resources can be used in the support of AI-enabled NGRA and SSbD case study work and its effective documentation including: (a) Implementing FAIR data principles; (b) Enabling effective retrieval of reliable well-referenced linked background knowledge organized into a knowledge graph; (c) Enhanced toxicology learning of Machine Learning- and Generative AI- based models; (d) Incorporation of AI knowledge into risk assessment and SSbD workflows and tasks, including analysis, evidence interpretation and decisions; (e) Generation of reports documenting workflow processes, including high integrity auditable supporting data and knowledge records; (f) Incorporating trust, interpretability and transparency; (g) Alignment with emerging AI guidance and legislation.

The presentation includes work carried out on several EU projects including EU-ToxRisk, RISK-HUNT3R, ASPIS, ACCORDs, SSbD4Chem and BioPhenom.

Presentation: Oral



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Occupational next generation risk assessment of the UV filters homosalate and 2-ethylhexyl salicylate

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Ensuring the safety of workers producing and handling chemicals is one of the main objectives of the European chemicals legislation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation. Information on the intrinsic hazards of chemicals is a key aspect in risk assessment, and those data are mainly derived from animal experiments. Interest in the application of alternatives to animal testing by using New Approach Methodologies (NAMs) is growing, main drivers being ethical reasons, an increasing need for more safety data due to a rapid development of new chemicals as well as the desire for more human-relevant data. The European ban of animal testing for marketing and safety assessments of cosmetic products and their ingredients triggered the development of Next Generation Risk Assessments (NGRAs). Lately, the applicability of NGRAs is being discussed also for substances regulated under REACH.

The UV-filters homosalate and 2-ethylhexyl salicylate (aka octisalate) are examples of cosmetic ingredients that have been safely used for many years. As they are manufactured in or imported into the EU, they are regulated under REACH, meaning any potential risk for the worker during handling of these substances needs to be evaluated and assessed which would necessitate further animal studies. To avoid this for cosmetic ingredients but nevertheless address all regulatory requirements, we performed NGRAs on the two substances using modelled as well as measured occupational exposure data. Physiologically based kinetic modelling was performed for *in vitro* to *in vivo* extrapolation. Developmental toxicity was specifically addressed in the calculation of Bioactivity Exposure Ratios (BERs) thus including also pregnant workers in the risk assessment. Comparing these results to conventional risk assessments conducted under REACH, equal conclusions could be drawn, i.e., handling of homosalate and 2-ethylhexyl salicylate during manufacture and formulation can be considered safe for all relevant use scenarios. This leads us to believe that no further animal experiments are needed to ensure worker safety.

Presentation: Oral

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Transition to xeno-free cell culture and organ models in research and teaching

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Non-animal methods (NAMs), such as organoids, bioprinted tissues, and multi-organ chips, are widely recognized as ethical and scientifically valuable alternatives to animal experiments. However, many of these approaches still rely on animal-derived components, including fetal calf serum (FCS), basement membrane extracts (BMEs, such as Matrigel), and gelatin. These materials not only raise ethical concerns but also contradict the goal of improving the translatability of research findings by introducing human cells into animal-derived environments, creating chimeric systems.

A recent systematic review on bioprinted liver models found no fully animal-free models [1]. To address this, we developed a completely xeno-free bioprinted liver model [2]. First, Huh-7 cells were adapted to a chemically defined, FCS-free medium. Next, we created a novel bioink that eliminates BME and gelatin, replacing them with non-animal materials. Additionally, cell culture procedures, including freezing and passaging, were adjusted to avoid animal-derived components. The resulting xeno-free liver model demonstrated high cell viability, strong structural integrity, and suitability for toxicity testing.

Recognizing that a true paradigm shift in biomedical research requires education, we developed an animal-free lab course. HeLa cells are cultivated in a chemically defined medium, which is not only animal-free but also more cost-effective than standard FCS-containing media. The course covers essential cell culture techniques, such as passaging, freezing of cells, transfections and RNA interference-based gene silencing, all performed without animal-derived materials. A Western blot is used to compare different antibody types (polyclonal, monoclonal, recombinant) and to discuss the potential of phage display-derived antibodies, which eliminate the need for animal immunization. The lab course script will be freely available, enabling universities worldwide to implement animal-free training.

We propose distinguishing between methods that avoid live animal use but still contain animal-derived materials, referred to as non-animal methods (NAMs), and fully animal-free technologies, which we term xeno-free methods (XFM). The commonly used term “new approach methodologies” is misleading, as it does not account for the replacement of animals or animal-derived components in biomedical research.

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Presentation: Oral



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Computer simulations supporting case-based teaching of experimental design

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Experimental design education aims to address ongoing scientific – and ethical – issues from poor reproducibility of both animal and non-animal research. Judging from systematic reviews of published literature, and reported failure to replicate preclinical results, fundamental principles, such as taking measures to avoid bias and determining the ability of a proposed experiment to detect a genuine effect, remain poorly understood by many researchers, despite the availability of textbooks and guidelines. As highlighted in Franco and Fry (2023, doi:10.1177/00236772221150299), interactive, problem-based teaching methods, including case-based discussions, have proved effective in fostering meaningful learning.

This talk will focus on the use of computer simulations as a tool to illustrate realistic experimental design scenarios, with which learners can relate. It will present a series of simulated experiments, using freely available software (G-Power, InVivoStat, spreadsheets) which help participants explore the impact of different design choices on statistical outcomes. Through step-by-step demonstrations, we will illustrate:

- The impact of small experimental group sizes on variability, false positive rates, and the ability to detect a genuine effect, in two group comparisons.
- The benefits of blocking as a strategy to increase efficiency and reduce the number of experimental units needed to detect an effect.
- The role of factorial designs, particularly in addressing the misconception that incorporating multiple variables (e.g., drug*sex*strain) necessarily requires large sample sizes.

When learners perform guided simulations where they manipulate parameters – e.g., group size, effect size, variability and different factors – they can directly observe their impact on research outcomes. By making these concepts visually accessible through software-generated graphs and power curves, researchers gain an intuitive understanding of experimental design principles that are often challenging when presented in a purely theoretical manner.

This case-based, problem-solving approach, with case studies that are representative of widely applicable experimental designs – for both animal and non-animal research – makes the teaching of the concepts more engaging and applicable, allowing learners to critically evaluate their own study designs and reduce common pitfalls in research planning, equipping researchers with the tools for designing robust, reproducible experiments while promoting the 3Rs. This talk aims to help trainers provide an “eye-opening” educational experience, and bridge the gap between theoretical knowledge and practical application in both animal and non-animal research.

Presentation: Oral

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Introduction to the 5Rs – A focus on refinement in DIT testing

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Traditional developmental immunotoxicity (DIT) testing has relied on the use of *in vivo* animal models, mainly pregnant rats, that were developed over 35 years ago. Toxicology has entered the New Approach Methodologies (NAMs) revolution; however, the field of DIT needs considerable research before NAMs can outright replace the need for *in vivo* models. Advances in current understanding of DIT modes of action and adverse outcome pathways (AOPs) will be critical for science-driven discovery and development of NAMs. *In vivo* DIT testing remains important to define modes of action and AOPs, however, DIT is in desperate need of refinement to improve predictivity and translatability of the data to human health risk assessment while at the same time improving animal welfare. Data-driven dose range selection represents an important refinement for *in vivo* DIT studies and can draw on non-animal data such as *in silico* read across, QSAR, and *in vitro* studies. Setting the dose range to high can lead to overt maternal toxicity that alters immune development through secondary effects. It is also critical to set the dose range in the context of human exposure as high dose toxicity can alter maternal metabolism and physiology leading to effects in the offspring that are likely irrelevant under real-world conditions, effects that could be misinterpreted as DIT. Another important refinement is the incorporation of disease models into DIT testing. For example, a drug intended to normalize blood glucose in pregnant diabetic women could lead to altered physiology and hypoglycemia in healthy pregnant women. Therefore, a significant refinement would be DIT testing for this drug in a diabetic rat model for improved relevance to the target human population. Improvements in our understanding of “normal” at all stages of immune development can lead to refinements in treatment protocols that reduce the duration of treatment with potential improvements in interpretation and animal welfare. This talk will provide a brief introduction to the 5 Rs for DIT and elaborate on refinements that show promise for increasing relevance, translatability, and prediction of *in vivo* models while at the same time representing refinements for improved animal welfare.

Presentation: Oral



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From data to decisions: AI's role in modern toxicology

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In recent years, artificial intelligence (AI) and machine learning (ML) have rapidly transformed toxicology, offering innovative approaches to predict chemical hazards, reduce animal testing, and accelerate regulatory decision-making. This talk will explore how AI/ML-driven models are being leveraged to augment human intellect and convert large toxicological datasets into actionable insights. By integrating high-throughput and high-content information with open-access computational toxicology workflows, we can now identify chemical hazards with unprecedented accuracy and efficiency. This talk will discuss how AI enhances our ability to automate reference data curation, manage large-scale datasets, develop predictive models, and ultimately support more informed decision-making within regulatory frameworks. Case studies will be used to illustrate AI's current applications in the field, its challenges, and its potential to shape a future where data drives safer and more sustainable chemical practices.

Presentation: Oral

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Moving from prioritization to risk assessment with NAMs: Canadian strategy to replace, reduce or refine vertebrate animal testing

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Health Canada is responsible for assessing and managing risks posed by chemicals to human health through defined acts and legislative frameworks. The traditional approach to risk assessment has primarily relied on hazard information derived from animal-based toxicity testing (e.g., rats, mice, fish) for a range of adverse health effects. These methods are low-throughput, resource-intensive and may be of limited biological relevance, presenting challenges to evaluating the vast number of chemicals in the human environment that require assessment. The recently amended Canadian Environmental Protection Act, 1999 (CEPA) recognizes the need to replace, reduce or refine the use of vertebrate animal testing when assessing the potential harms that substances may pose to human health and the environment. In response, the Government of Canada has developed a strategy to guide efforts to develop, standardize and incorporate New Approach Methods (NAMs) into hazard and risk assessment activities. Here, we provide an overview of the elements of the strategy, and highlight some areas that Health Canada is actively investigating to reduce reliance on animals for toxicity testing, while addressing data needs for risk assessment.

To support an iterative transition, a range of alternative assays and technologies are being explored to strategically advance the use of NAMs for human health risk assessment. Science approach documents (SciADs) describe NAMs that are starting to be used for prioritization and assessment of existing substances under CEPA. Examples include, (1) the Bioactivity-Exposure Ratio (BER) Approach demonstrating the use of high throughput bioactivity assays to generate points of departure for regulatory application; and (2) the Health Canada Automated Workflow for Prioritization, an integrated computational tool that incorporates various types of biological data from *in silico* predictions, *in vitro* and *in vivo* assays into a logical decision-making framework. The application of transcriptomics and gene expression profiles are being implemented to expand on the use of *in vitro* assays. Transcriptomics continues to be an area of focus to develop best practices and interpretation workflows to build confidence for the derivation of practical points of departure and reveal areas of remaining uncertainty that warrants further work. These initiatives will be highlighted to demonstrate the opportunities for NAMs to effectively aid in various decision-making contexts under CEPA.

Overall, the strategy helps to guide and promote the development and adoption of scientifically justified alternative approaches for toxicological testing that align with current domestic and international efforts.

Presentation: Oral



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One Health case studies: Bridging human and ecotoxicology through cross-species comparative approaches

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The One Health framework highlights the need for mechanistic approaches that integrate human and environmental toxicology. While human toxicology benefits from abundant high-throughput screening (HTS) and mechanistic data, ecotoxicology often lacks standardized *in vitro* datasets, making cross-species extrapolation essential for assessing chemical hazards in wildlife. This study demonstrates how molecular docking and bioinformatics approaches can leverage human toxicology data to predict chemical susceptibility in endangered marine turtles.

We first identify endocrine-disrupting plastic additives using Tox21 nuclear receptor bioassay data and molecular docking simulations for human receptors. To extend these findings to marine turtles, we apply cross-species extrapolation techniques, including sequence conservation analysis (SeqAPASS) and species-specific molecular docking, to evaluate receptor susceptibility. By comparing binding affinities between human and marine turtle receptors, we assess the likelihood of endocrine disruption in these vulnerable species.

Additionally, we explore the implications of these findings in the context of chemical risk assessment. By linking human-relevant *in vitro* bioactivity data with potential adverse effects in marine turtles through cross-species adverse outcome pathway (AOP) networks, we demonstrate how mechanistic modeling can fill knowledge gaps in ecotoxicology. This approach provides a science-based framework for prioritizing chemicals of concern in wildlife conservation and regulatory decision-making.

By systematically extrapolating human toxicity findings to endangered species, this study illustrates how cross-species molecular docking can enhance ecotoxicological predictions, reduce reliance on traditional animal testing, and strengthen chemical risk assessment under the One Health paradigm.

Presentation: Oral

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Recommendations from an ECETOC workshop to advance the application of AI in (eco)toxicology

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The long-term investment in new approach methodologies (NAMs) has resulted in an emerging consensus on how to use information from *in silico*, *in vitro* and *in vivo* sources to assess the safety of chemicals. An ECETOC Task Force has developed a framework incorporating *in silico*, *in vitro* and *in vivo* methods designed to meet regulatory requirements in which both hazard identification, classification and exposure can be assessed using a tiered approach (Ball et al., 2022). Although this framework considered *in silico* information, the rapid development of machine learning and AI in the last two years promises new unrivaled opportunities as well as significant challenges. To assess the state of the art of the application of AI in chemical safety assessment and to develop guidance on how to make best use of data, ECETOC organized a workshop to leverage the collective expertise of scientists from academia, regulators and industry. The major recommendations from this workshop are associated with the data and building trust in AI for its use in a regulatory context.

Data availability can be increased by reducing barriers for data sharing or even providing incentives for doing so. This may be achieved the “honest broker” concept, which has been used for data sharing within the pharmaceutical and agricultural industries. It is essential that data are provided in a machine-readable format and with sufficient meta data. Data quality can be addressed by a scoring system and automated if sufficient meta data are available. FAIR criteria and the storage / maintenance of databases should be an integral and mandatory part of all (inter)nationally funded projects.

Building trust in the use of AI requires clear data source identification, and the avoidance of generative AI. It also requires an understanding where AI can be used and how (applicability domains). AI outcomes should be assessed by experts to ensure that the results sense. The use of AI for regulatory purposes would require an active role of international organizations ensuring organizational impartiality (e.g., OECD)

Reference

Ball et al. (2022). *Arch Toxicol* 96. doi:10.1007/s00204-021-03215-9

Presentation: Oral



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ANVISA and Brazilian Pharmacopoeia's perspective on non-animal testing regulatory acceptance

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In Brazil, the National Health Surveillance Agency (ANVISA) is the Brazilian regulatory agency that is responsible for regulating, approving and monitoring the quality control of medicines including biologicals products, among which vaccines. ANVISA and the Brazilian Pharmacopoeia have started an important work towards introducing alternative methods to the use of animals, in alignment with the agenda of the National Council for the Control of Animal Experimentation (CONCEA), and also aligned with the latest changes in the field promoted by other regulatory agencies and organizations as World Health Organization (WHO), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and recognized Pharmacopoeias. This presentation will provide an overview of the regulatory changes introduced by ANVISA and by the Brazilian Pharmacopoeia, like the complete deletion of the Abnormal Toxicity Test, the creation of a chapter dedicated to the Monocyte Activation Test to detect pyrogens, the revision of vaccine monographs in progress and share the other changes that are currently under consideration. This presentation will also highlight the challenges that ANVISA, official laboratories and drug manufacturers needed to overcome to secure the introduction of those changes in the regulations and manufacture and how international collaboration has been key element to promote such changes.

Presentation: Oral

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Regulatory acceptance of alternatives to animal testing for biological products

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The Brazilian Health Surveillance Agency (Anvisa) is the Brazilian Regulatory Agency responsible for approving and monitoring medicines exposed to consumption in Brazil. The company responsible for the regularization of medicines must have complete data proving the quality, efficacy and safety of the product and guarantee that the product complies with current regulations. According to Resolution RDC n° 948 of 2024, the term medicine applies to the pharmaceutical product technically obtained or prepared, with prophylactic, curative, palliative or diagnostic purposes. This regulation requires that quality control tests for active pharmaceutical ingredients and finished products may use official pharmacopoeial methods or internal development methods (in-house), provided they are duly validated under the terms of the Resolution RDC n° 166, from 2017, and its updates. The pharmacopoeial methods contained in the Brazilian Pharmacopoeia or in recognized Pharmacopoeias, in accordance with Resolution RDC n° 511, from 2021, or its updates, are considered official. When adopting internal development methods, these must be equivalent or superior to those of the Brazilian Pharmacopoeia, if they exist, and may be accessed, assessed and incorporated by Brazilian Pharmacopoeia, unless expressly refused by the company holding the regularization. The possibility of using methods from recognized Pharmacopoeias and validated internal development methods provides the possibility of introducing innovative methods, including alternative methods to the use of animals. The 1988 Federal Constitution, by imposing the duty to protect and preserve the environment and prohibiting, in accordance with the law, practices that cause the extinction of species or subject animals to cruelty (Art. 225), supports the use of alternative methods in Brazil. ANVISA's Resolution RDC n° 35, from 2015, declares the acceptance of alternative methods in petitions submitted for analysis through the National Council for the Control of Animal Experimentation (CONCEA), except in specific cases in which ANVISA, through technical justification, presents the inadequacy and inapplicability of the methods recognized by CONCEA. Procedures for including new methods in the Brazilian Pharmacopoeia are time-consuming, since the methods are evaluated and written by Thematic Technical Committees of the Brazilian Pharmacopoeia, including representatives from universities, official laboratories, drug manufacturers and ANVISA, afterwards the regulatory impact assessment must be carried out and, subsequently, the new methods undergo Public Consultation so that society could assess their suitability. The presentation will give an overview of the Regulatory Acceptance of alternative methods to the use of *in vivo* methods for medicines in Brazil.

Presentation: Oral



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Non-animal toxicity testing strategies in China: Development and challenges

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Toxicity testing is an important approach for safety assessment of chemicals such as environmental agents, drugs, food-additives and cosmetics, providing evidence and protection for human health and environmental safety. It's imperative to develop alternative testing methods and translational toxicology research of human related markers and approaches. In 2007, the U.S. National Research Council released a key report, Toxicity Testing in the 21st Century: Vision and Strategies (TT21C). Since then, new approach methodologies (NAMs) for toxicological testing have emerged for assessing chemicals and products like pharmaceuticals, food, cosmetics, and pollutants. These methods include *in vitro*, *in chemico*, and *in silico* models, and integrated strategies that study human biology more efficiently and with fewer animals. Although the development and application of alternative *in vitro* methods for toxicity testing in China is slow as compared with the advanced countries, efforts and some progresses in this field have been made in recent decades. In 1997, four Chinese administrative ministries proposed the first development plan for alternative methods in China linked to 3Rs principles of laboratory animal sciences. Subsequently, a series of measures have been taken to promote the development and regulatory application in China, including uniting scientists from academic institutions, industries and governmental agency, construction of technological platforms for development of alternative methods, setting relevant scientific programs, conduct of continuing education and training to publicize the significance and application of alternative methods, and the formulation of relevant technical guidelines for regulatory uses in cosmetic safety, chemical safety, and environmental safety. Particularly, during the past decade, a number of scientists have participated in introducing TT21C to the government, industrial and academic circles by establishing scientific societies, holding symposia and organizing continuing education training programs. A new scientific society was established in China in 2014, which is the Chinese Society of Toxicological Alternatives & Translational Toxicology (TATT) under the China Society of Toxicology (CSOT). The establishment of TATT has marked another milestone in China's commitment to the development of non-animal approach for assuring safety and risk assessment. This society provides useful scientific platforms with a focus on drawing together both domestic and international efforts to promote the new safety science as well as to facilitate acceptance of this new science in potential regulations of the future. This present talk will highlight significant progress from TT21C to NAMs developments in China over the past decade and including the progresses for building testing methods based on perturbations of toxicity pathway in our lab as a model for demonstrating the new strategy of new generation risk assessment (NGRA).

Presentation: Oral

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Status and future outlook of non-animal testing methods in South Korea

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The landscape of alternative testing methods in South Korea has evolved significantly since the establishment of the Korean Society for Alternative to Animal Experiments (KSAAE) in 2007 and KoCVAM in 2009. This development reflects global trends toward reducing animal testing while ensuring safety assessment reliability, while incorporating unique Korean approaches to 3Rs implementation.

The current status of non-animal testing methods in South Korea and future perspectives focus on regulatory frameworks, technological advancements, and international collaboration networks, with distinct characteristics reflecting Korean research and industrial environment.

South Korea has made substantial progress in implementing alternative testing methods, marked by the 2017 ban on animal testing for cosmetics and the establishment of policies supporting alternative method development. The KSAAE, in collaboration with KoCVAM, has been instrumental in advancing these initiatives through regular academic publications, workshops, and international conferences.

Unique aspects of 3Rs dissemination in Korea include:

- Emphasis on K-beauty industry requirements and standards
- Close collaboration between government, academia, and industry through unique consortium models
- Development of specialized training programs in collaboration with regulatory authorities
- Active participation of Korean biotech startups in alternative method development

South Korea is well-positioned to continue advancing non-animal testing methods through its established infrastructure, regulatory support, and international partnerships, while maintaining its distinctive approach to 3Rs implementation. Future success will depend on sustained commitment to technological innovation, regulatory harmonization, and global collaboration, while preserving and developing Korea-specific solutions and methodologies.

Presentation: Oral



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Bioprinting skin tissue models for toxicology evaluation

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The application of light-based technologies for recapitulating skin layers presents a promising approach for drug screening and testing. Traditional *in vitro* models often fail to fully replicate the complex architecture and functionality of human skin, which limits the accuracy of drug efficacy and toxicity predictions. This study explores the potential of light-based technologies to generate three-dimensional (3D) skin tissue models, mimicking the epidermis, dermis, and hypodermis layers with high precision. By utilizing advanced light projection and patterning techniques, we can construct skin organoids and multilayered skin models that more closely resemble native human tissue. These engineered skin structures provide a platform for drug screening, offering advantages such as better drug absorption, skin barrier function, and cellular responses. The integration of light-based technologies in skin model development allows for high-throughput testing, reducing reliance on animal models while enhancing the predictability of human skin responses to pharmaceutical compounds. This innovative approach has the potential to significantly improve the accuracy and efficiency of pre-clinical drug testing, advancing the field of dermatopharmacology and personalized medicine.

Presentation: Oral

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One Health approach by transdisciplinary and exploratory research in Japan

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It is becoming clear that human activity is causing disturbances to the global environment and ecosystems, such as global warming and the emergence of antibiotic-resistant bacteria. In contrast, there are concerns that the transformation of the global environment and ecosystems caused by human activity will lead to the development of diseases and prevent us from achieving a healthy and long-lived society. Therefore, human health cannot be considered in isolation from human society, and it is necessary to establish the principles of “One Health”, which takes an interdisciplinary approach to the health of the entire planet, including humans, animals and plants.

Based on collaboration with experts from academia, industry and regulatory agencies from various fields, we have discussed One Health as a government project. The aim of this project is to promote collaboration of various fields with the new technology NAMS to predict the interactions and effects between living organisms, including humans, and the environment. Specifically, we will hold a cross-disciplinary symposium on One Health, and share information with related academic organizations, companies, and citizens, and promote organic collaboration through introducing NAMS on “homeostasis (maintenance and disruption of biological homeostasis)”, “exposome (interaction with the environment)”, “sensing (environmental perception and response)”, and “digital (prediction of pre-symptomatic disease, countermeasures against emerging infectious diseases)”.

Here I would like to talk about our activities and future perspectives on One Health.

Presentation: Oral





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Animal-free induced pluripotent stem cell-derived microvascular models to replace the use of mouse models in vascular research

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Currently, microvascular disorders such as Spontaneous Coronary Artery Dissection (SCAD), lack appropriate three-dimensional (3D) modelling systems beyond animal models. For sufferers of SCAD, the cause of approximately one third of heart attacks in women under 50, and the primary cause of heart attacks in pregnant women, the lack of tools, techniques and knowledge have resulted in a poor understanding of the primary causes of SCAD. As a consequence, there are currently no specific treatments available to patients, despite a high rate of recurrence (up to half of SCAD patients will have a repeat heart attack), and heritable risk factors which put future generations at risk. Here, we demonstrate the first step towards addressing this unmet need; spontaneously vascularizing environments, incorporating cellularly complex, functional microvasculature within 3D printed devices – a state-of-the-art approach for studying blood vessel development and growth within confined microfabricated environments. Using animal product replacements provides a robust and more biologically relevant animal-free system to study the disease mechanisms of SCAD, as well as other microvascular-affecting diseases including atherosclerosis and diabetes.

Presentation: Oral

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Reduction: Minimizing animal use in DIT research

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Developmental immunotoxicity (DIT) refers to the adverse effects of chemical, biological, or environmental exposures on the developing immune system, particularly during prenatal, neonatal, or early postnatal life. Given the immune system's heightened vulnerability during these critical windows, exposure to immunotoxic agents can lead to long-term or irreversible dysfunction.

Currently, the only accepted methods for assessing DIT rely on *in vivo* testing. The OECD Test Guideline 443 (Extended One-Generation Reproductive Toxicity Study) includes Cohort 3 specifically for evaluating chemical impacts on immune development. However, this method requires an average of 1,826 animals per tested chemical, increasing to 2,734 if the study is extended to a second generation (Knight et al., 2023). Approximately 508 of these animals are used for the dose range-finding (DRF) test alone. While New Approach Methodologies (NAMs) hold promise for future DIT assessments, there is an urgent need to reduce animal use in the interim.

Toxicological studies typically include four groups of animals: a control group and three dose-exposure groups (low, medium, and high). Each study generates new control data, adding to extensive historical datasets housed within testing facilities. The VICT3R project (Developing and Implementing Virtual Control Groups To Reduce Animal Use in Toxicology Research) proposes leveraging these historical control datasets, combined with Artificial Intelligence (AI), to establish virtual control groups (VCGs) that could replace live control animals. If fully implemented, this approach has the potential to reduce animal use by 25% in DIT studies.

VICT3R, a public-private partnership funded by the Innovative Health Initiative (IHI) Joint Undertaking under grant agreement No. 101172693, aims to generate the scientific evidence necessary to support the regulatory adoption of VCGs. By aligning with the 3Rs principles (Replacement, Reduction, and Refinement), this initiative seeks to enhance the ethical, scientific, and economic efficiency of toxicology research while advancing regulatory acceptance of AI-driven methodologies.

The first step in VICT3R is collecting historical data from concurrent control groups (CCGs). Agreements with pharmaceutical companies are in place, and Contract Research Organizations (CROs) will further expand the dataset. These data require thorough curation and standardization, as formats vary. Quality checks will identify inconsistencies and outliers. The dataset primarily includes rats and mice, with some data from dogs and non-human primates (NHPs). Key variables include strain, age, administration route, organ weights, and histopathology, though some studies may lack complete data. AI-driven approaches will then analyze statistical patterns to generate Virtual Control Groups (VCGs). Before replacing concurrent controls, VCGs must be validated to ensure reliability and define uncertainties. Regulatory experts are actively involved, guiding data collection and database development. Given the complexities of Good Laboratory Practice (GLP) studies, initial applications will focus on non-GLP research and early-stage tests, such as Dose Range-Finding (DRF) studies, before broader regulatory adoption.

Presentation: Oral



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From molecules to ecosystems: Computational toxicology for sustainable and safe cosmetics

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The cosmetics industry is undergoing a significant transformation driven by increasing consumer demand for safer and more sustainable products. This transformation requires moving beyond traditional animal testing methods and embracing innovative approaches like computational toxicology, with *in silico* approaches emerging as powerful tools to predict potential human and environmental hazards. Computational toxicology integrates advanced modeling techniques, such as [quantitative] structure-activity relationship ([Q]SAR/SAR) models, machine learning algorithms, and physiologically based pharmacokinetic (PBPK) modeling, to evaluate toxicity profiles without relying on new animal testing. We will be presenting the crucial role of *in silico* tools in bridging the gap between molecular interactions and ecosystem impacts, enabling the development of cosmetics that are both safe for humans and environmentally responsible. The use of *in silico* predictions for regulatory purposes still raises questions and uncertainties. However, these tools enable the assessment of various endpoints, particularly local exposure, which are relevant to the cosmetics landscape. They contribute to a weight-of-evidence approach when combined with other data, providing robust information for the development of safer products for consumers. Similarly, the development of products with reduced environmental impact has become a commitment within the cosmetics industry. In this context, *in silico* tools are employed to predict the potential for biodegradation, bioaccumulation, aquatic toxicity, and physical-chemical properties, especially in cases where empirical data is unavailable or existing studies are unreliable. These predictions are used in our risk assessment matrix called *Índice de Avaliação de Risco Ambiental* (IARA) (Environmental Risk Assessment Index). IARA compiles diverse information, including *in silico* predictions, physicochemical properties, GHS classification, endocrine disruption potential, and other data on ingredients used in various product types. Based on this information, we assign environmental impact scores to each ingredient and subsequently evaluate the overall impact of the products. In summary, this study highlighted the power of computational toxicology in driving the future of cosmetics. These tools complement *in vitro* and literature data, particularly for complex endpoints, and offer valuable pre-screening for new substances. However, further development of tools and databases specifically tailored to cosmetic ingredients is necessary to enable more reliable predictions.

Presentation: Oral

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Integrating human and environmental mechanistic data to support safety decisions: Identifying synergies and potential challenges

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This presentation aims to delve into opportunities for breaking the silos and building bridges between human and environmental next generation risk assessment. We will discuss the opportunities arising from the evaluation of mechanistic-based data, namely the use of New Approach Methodologies (NAMs).

A novel framework has been designed to set out common ground while recognizing the inherent characteristics of each field, enabling a common language and ensuring enhanced protection of both consumers and the receiving environment. The presentation will emphasize the similarities, such as internal exposure and toxicological conservation across species, as well as the differences, including protection goals and problem formulation. In line with the next generation risk assessment concept, the focus will be on identifying synergies that can enhance the reliability and applicability of safety decisions across various contexts under the One Health concept. Additionally, we will address the potential challenges associated with integrating and extrapolating mechanistic data across human and environmental studies due to their evolutionary distance. Overall, this presentation seeks to provide a deeper understanding of the implication of applying such data in regulatory frameworks and to explore strategies for overcoming obstacles on the path towards more robust transparent and structured safety assessments, leveraging the wealth of data available.

Presentation: Oral



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Genomic and proteomic characterization of patient-derived breast cancer organoids from South-Eastern Bantu patients

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Cancer care for African women is based on data gathered in the global West, resulting in biased treatment results due to their underrepresentation. This bias has real consequences in adverse effects of treatments, how treatment regimens are informed as well as the paucity in understanding cancer biology in this underrepresented population. A major limitation of African-centric cancer care is the lack of appropriate pre-clinical model systems for investigation. This project aimed to establish a panel of patient-derived breast cancer organoids from African patients with a view to identify markers that can be used to assist in chemotherapy treatment decisions and to identify novel genetic alterations and physiological differences that may inform diagnostic and treatment decisions.

Resected tumor tissue obtained from mastectomies was collected and processed to establish organoid lines. Established lines were exposed to the chemotherapies currently available in the public health system (doxorubicin, paclitaxel and 5-fluorouraci). Next generation sequencing was performed to uncover variants in 50 cancer-specific genes. The migratory phenotype of the different lines was also assessed through adhesion and migration assays.

Multiple organoid lines originating from clinically diverse tumor types have been established, expanded and assayed. Drug response experiments reveal distinct differences in sensitivity of organoid lines to chemotherapy treatment. Genomic analysis of 50 cancer gene panel revealed that variants present in original tumor tissue is mostly retained in the organoid lines, indicating the utility of this as patient-specific preclinical model. Phenotypic characterization revealed that organoids are diverse in their adhesive and spreading capability, with some lines displaying a complete lack of spreading capability while others have migratory phenotypes, suggesting increased metastatic potential. With the establishment of a small African breast cancer organoid panel, we have created a resource that can be utilized when investigating breast cancer biology in African patients and in the development of African-centric cancer care strategies.

Presentation: Oral

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Overcoming data gaps: Gaining acceptance for NAMs and DAs in case of limited applicability domain

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The integration of New Approach Methodologies (NAMs) and Defined Approaches (DAs) into regulatory frameworks for hazard assessment depends on their ability to provide reliable and relevant information. A significant obstacle to their widespread adoption is the limitation of their Applicability Domains (ADs). This presentation addresses the challenges of gaining acceptance for NAMs and DAs when data is limited and ADs are restricted, drawing on the development and evaluation of a Defined Approach for Surfactants (DASF) as a case study.

The DASF, designed to assess eye hazard potential according to UN Globally Harmonized System (GHS) categories, combines *in vitro* Reconstructed human Cornea-like Epithelium (RhCE) test methods with a modified Short Time Exposure (STE0.5) assay.

A critical aspect was establishing a robust and transparent process for selecting reference chemicals. Given the restricted AD (surfactants), careful consideration was given to relevant drivers of classification, leveraging historical *in vivo* Draize eye test data. Recognizing the limited availability of high-quality *in vivo* data for surfactants, a tiered approach was adopted. This involved:

- A comprehensive analysis of existing *in vivo* data to identify key drivers of classification and their relative importance for each GHS category.
- A targeted effort to fill data gaps by expanding the reference chemical set.
- Prioritizing chemicals with well-characterized properties and mechanisms of action to enhance confidence in the extrapolation of *in vitro* results to *in vivo* hazard.

Key lessons from the DASF case study for gaining acceptance of NAMs and DAs with limited ADs include:

- Establishing transparent and relevant performance metrics (sensitivity, specificity, balanced accuracy), agreed upon by expert groups.
- Clear and transparent documentation of the rationale for the chosen AD, including a detailed analysis of the available data and a justification for all statements made.
- Focus on Mechanistic Relevance: Prioritizing *in vitro* methods that capture key mechanisms of toxicity relevant to the hazard being assessed, enhancing confidence in the biological plausibility of the NAM/DA.
- Adaptive Testing Strategies: Optimization of the STE test method to better discriminate between Cat 1 and Not Cat 1.
- Open Communication: Promoting ongoing dialogue and collaboration with stakeholders (e.g., regulatory agencies, industry, and research institutes) to address concerns, share data, and build confidence in the NAM/DA.

By addressing these key challenges and embracing a transparent and rigorous approach, NAMs and DAs can gain wider acceptance, ultimately expecting an OECD adoption in the respective Test Guidelines (TG): TG 491 for the STE0.5 and TG 467 for the DASF for eye hazard identification of chemicals with surfactant properties.

Sharing these key learnings may also play a critical role in developing better testing and assessment strategies for any NAM/DA, supporting the paradigm shift away from animal testing across various endpoints.

Presentation: Oral



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Replacing fish experiments with fish cells grown in animal-free cell culture medium

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In vitro fish cell culture has emerged as a promising alternative to whole-animal experimentation in toxicology and biotechnology. Among the most widely applied fish cell lines, the rainbow trout gill cell line (RTgill-W1) has been instrumental in advancing standardized toxicity testing, culminating to its adoption in OECD Test No. 249 and ISO Standard 21115. However, while this assay now serves as replacement for the fish acute toxicity test, the last remaining bottleneck is the continued reliance on fetal bovine serum (FBS) as an essential supplement for routine cell growth and proliferation. FBS is an undefined, animal-derived component, raising both ethical concerns and scientific challenges, including batch-to-batch variability and potential interference with chemical testing.

To address this limitation, we developed a systematically optimized serum-free medium that supports long-term RTgill-W1 cell proliferation while maintaining functional equivalency to FBS-supplemented cultures. Our approach involved an iterative process of targeted medium component selection, high-throughput screening using a 96-well plate proliferation assay, and stepwise adaptation of RTgill-W1 cells to serum-free conditions. The optimized medium supports robust cell growth, metabolic activity, and morphology comparable to FBS-containing medium. Furthermore, as judged from a proof-of-concept using the positive control chemical, 3,4-Dichloroaniline, cells maintain their functionality in OECD TG249, demonstrating the suitability of the serum-free medium for regulatory toxicity testing applications. However, a key challenge in designing this medium was optimizing albumin and lipid supplementation. Our findings indicate that selecting an appropriate albumin source is more complex than initially perceived. Several types of albumin supplementation were tested for their impact on the short-term cell proliferation: bovine serum albumin (BSAreg) and recombinant human serum albumin (rHSA) expressed either in *Oryza sativa* or *Pichia pastoris*. The results showed that rHSA had a negative impact on RTgill-W1 cell viability, regardless of the source. Recombinant albumins represent a promising direction for fully defined culture conditions, but further refinement is needed. Most of the components in our serum-free formulation originate from a commercially available lipid mixture containing 13 distinct compounds. Our preliminary data suggest that downsizing the lipid mixture to only two essential fatty acids and recombinant cholesterol supports equivalent cellular viability and proliferation compared to the full lipid formulation.

RTgill-W1 cells cultured in this fully defined medium have been successfully passaged over 35 times, cryopreserved, and recovered without loss of viability. This work marks the first report of a serum-free medium for long-term culture of a fish cell line and lays the foundation for a completely animal-origin-free medium. Future efforts will focus on testing if other rainbow trout cell lines could be cultured similarly in this serum-free medium.

Presentation: Oral

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The impact of ethical review on 3Rs implementation: An assessment of Canadian animal research

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The use of animals in research is justified by the expectation that the scientific benefits will outweigh the harms to the animals involved. In Canada, ethical review committees evaluate and approve animal use protocols (documents that describe and justify planned research), conducting harm-benefit analyses to ensure this standard is met. These committees can request additional information or modifications to enhance research benefits (e.g., ensuring sample sizes are sufficient for statistically valid findings) and to minimize harms through the implementation of the 3Rs (replacement, reduction, and refinement). However, evidence suggests there is room for improvement.

Evaluations of published animal studies reveal that even well-established 3Rs practices are not routinely implemented (e.g., use of evidence-based refinements like non-aversive animal handling remains uncommon). Likewise, most studies do not adopt rigorous methods (e.g., randomization), increasing the risk of bias and undermining potential benefits. Further, qualitative studies have revealed incomplete understanding of the 3Rs within ethical review committees and a bias toward scientific and institutional values. Yet reporting within Canadian animal use protocols has never been investigated. We therefore sought to assess protocols approved by ethical review committees to identify areas for improvement.

In partnership with Canada's oversight body for animal science, the Canadian Council on Animal Care (CCAC), we conducted an assessment of animal use protocols to characterize reporting of 1) the 3Rs; and 2) rigorous methodology (randomization, blinding, sample size calculation, planned data analysis). A request for submission of animal use protocols was sent to 35 academic institutions. From each institution, we requested the last 3 mouse and 3 rat protocols approved prior to January 1, 2024, for minimal, low, moderate and severe stress categories of invasiveness, in both basic and translational research (n = 48 protocols/institution). Screening and data extraction were performed independently and in duplicate.

Fifteen institutions participated (43%), submitting 308 protocols in total (60% mice, 30% rats, 10% both species). Most protocols involved basic (53%) or translational research (35%), with 3% involving both, and 6% unclear. For categories of invasiveness, 25% of protocols were minimal stress, 36% low, 37% moderate, 1% severe and 1% unclear. Replacement of animals was described in 29% of protocols, and 65% explicitly justified use of animals. Efforts to reduce animal numbers were reported in 75% of protocols, and refinements in 39%. With respect to rigorous methodology, blinding was reported in 7% of protocols, randomization in 20% and planned data analysis in 16%. Use of sample size calculations was reported in 28% of protocols, of which 17% provided calculation details (e.g., effect size, power).

These results represent the first characterization of reporting in Canadian animal use protocols. They highlight current strengths, such as



reporting of replacement and reduction, but also reveal areas of incomplete reporting. Together, these findings will help “evidence-inform” updates to the CCAC’s Guidelines on Animal Use Protocol Review. Our next steps include a focus group study with ethical review committee members to explore barriers and enablers to requiring reporting of the 3Rs and rigorous methods, and adherence to CCAC guidelines.

Presentation: Oral

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Animal-free safety assessment of chemicals – An innovation systems perspective

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Transitions, including the transition towards animal-free innovation, are complex and often chaotic processes. To grasp the systemic nature of the changes needed to accelerate animal-free innovation, an innovation system approach is proposed. This is a heuristic framework for understanding and appraising mission achievement, which we applied to the case of animal-free chemical safety assessment. Using this approach, we propose that changes in this innovation system are not only dependent on technological advancements of new approach methodologies (NAMs) but also broader socio-institutional shifts in mindsets, regulatory structures and congruent hazard and risk assessment practices. The innovation system approach describes interdependent key processes that need to be fulfilled to achieve the mission of animal-free chemical safety assessment. Besides the more commonly acknowledged processes like Research & Development, Validation and Acceptance, there are other important key processes such as Resource Mobilisation, Experimentation (both in companies and in regulatory settings), legitimacy creation, and mission governance. The innovation system approach puts emphasis on new ways of evaluating and monitoring progress as well as on how to formulate, coordinate and guide towards achievement of the mission of animal-free chemical safety assessment.

Presentation: Oral

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Skin tissue organoids for toxicology and drug evaluation

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Skin tissue organoids hold significant potential for tissue engineering, drug screening, and toxicology studies. Recent advances in skin cancer models have shown great promise regarding drug efficacy testing. Melanoma, an aggressive form of skin cancer, poses a significant public health challenge due to its high mortality rate and increasing prevalence. Traditional two-dimensional (2D) cell cultures, while valuable in the evaluation of biological processes, fall short in replicating the complex tumor microenvironment in melanoma, limiting their predictive power for *in vivo* drug responses. The development of three-dimensional (3D) cell culture models has shown promise in providing a more biologically relevant representation of the TME.

To address this gap, this study developed a simple and highly reproducible method to fabricate and analyze 3D bioprinted A375 melanoma cell culture models. Utilizing a hydrogel biomaterial and bioprinting technology to create reproducible cell-laden construct for drug efficacy testing. This approach enabled precise quantification of apoptosis and cell viability in physiologically relevant 3D bioprinted melanoma cell culture models. To optimize this method, the bioprinting parameters for producing 3D cell cultures were optimized to ensure reproducibility while maintaining the desired construct size and preserving cell viability. After optimization of the bioprinting process, the A375 melanoma 2D and 3D bioprinted cell cultures were assayed for drug-induced apoptosis through a flow cytometric and a colorimetric resazurin-based analysis method and compared to determine the efficacy of the proposed flow cytometric analysis method.

Compared to the 2D cell cultures, the 3D bioprinted cell cultures showed higher levels of resistance to the chemotherapy drugs evaluated, as demonstrated by the higher IC50 values of the 3D cell culture models. As a result, the 3D bioprinted melanoma model provides a reproducible, simple, and effective approach for fabricating and analyzing 3D bioprinted melanoma cell culture models without introducing complexity to existing assays. It holds significant potential for enhancing the evaluation of drug efficacy in 3D melanoma models with skin tissue organoids, contributing to more accurate and reliable outcomes in toxicology and drug efficacy testing.

Presentation: Oral



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Progressing IATA for systemic toxicity: Guidance to advance the evaluation of new approach methodology (NAM)-based approaches for systemic toxicity

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The identification of New Approach Methodology (NAM)-based solutions to assess systemic toxicity has been identified as a high priority for OECD member countries generally and for the OECD Working Party on Hazard Assessment (WPHA), in particular.

Next-Generation Risk Assessment (NGRA) is an example of a NAM-based approach which uses a general framework to guide the hypothesis-driven safety assessment of chemicals or ingredients in products. A key feature of this approach is to use NAMs in concert to assess the internal exposure and potential toxicity of a particular ingredient for a defined use. A number of case studies have used this approach to assess the safety of prototypical ingredients, and data for many chemicals using a range of NAMs approaches has been generated.

This OECD project aims to develop guidance, case studies, analyses, and other collaborative outputs to advance describing, and applying, an evaluation framework for emerging NAM-based methods to support their regulatory use as part of Integrated Approaches for Testing and Assessment (IATA) for Repeat-Dose Systemic Toxicity.

This presentation will highlight project progress within the OECD WPHA Expert Group and highlight several case studies that allow for derivation of points of departure that are broadly protective for systemic toxicity.

Presentation: Oral

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Hands-on training for the implementation and consolidation of NAMs in Brazil

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The approval of Law No. 11,794/2008, known as the Arouca Law, was a milestone for regulating animal use in scientific experimentation and education in Brazil and Latin America. This law led to the creation of the National Council for the Control of Animal Experimentation (CONCEA), which oversees the introduction of alternatives to animal testing. In 2012, the Brazilian National Network of Alternative Methods (RENAMA) was established with 51 members. RENAMA focuses on developing, validating, training, and disseminating new approach methodologies (NAMs) to reduce dependence on external technology. In 2015, the Regional Platform of Alternative Methods to the Use of Animals (PReMASUR) was created, relying on RENAMA for education and training activities in Argentina, Brazil, Paraguay, and Uruguay. Moreover, CONCEA No. 58/2023 imposed a partial ban on cosmetic testing on animals, prohibiting the use of vertebrate animals in the scientific research, development, and quality control of cosmetics. Most recently, in November 2024, Brazil enacted Law 15,022, which establishes the National Inventory of Chemical Substances and sets regulations for the risk assessment and control of chemicals produced or imported into the country. This presentation will highlight educational activities empowering innovators and regulators to apply modern research and regulatory assessment approaches. For example, the Laboratory of Education and Research in *In vitro* Toxicology at the Federal University of Goiás, Brazil, has introduced new *in vitro* scientific concepts to hundreds of undergraduate and graduate students. Our team has trained professionals, scientists, and regulators from Brazil and other South American countries in non-animal methods through in-person lectures, networking opportunities, online webinars, and hands-on demos. By working cooperatively, we have demonstrated how non-animal safety science is best used to regulate the ethical use of chemicals, enabling sustainable innovation and ensuring human and environmental safety. We have focused on cross-sectoral collaboration with academia, industry, CROs, and regulators to advance knowledge of NAM-based chemical assessments throughout Brazil and Latin America, assisting researchers and communicating advanced NAMs to accelerate adoption and regulatory acceptance.

Presentation: Oral



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Including interindividual variability in acute reference dose setting for diazinon using population physiologically based kinetic and toxicodynamic (PBK-TD) modeling

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Organophosphate (OP) pesticides are widespread in the environment, resulting in human exposure. The risk of acute neurotoxicity in humans through acetylcholinesterase (AChE) inhibition remains a major public health concern. However, not all humans are equally sensitive to the OP-induced AChE inhibition. The aim of the present study was to quantify this variable susceptibility of the general adult population following acute OP exposure. To this end, a population physiologically based kinetic and toxicodynamic (PBK-TD) model was established for diazinon (DZN), an exemplary OP pesticide. The population PBK-TD model was parameterized with physiological, toxicokinetic and toxicodynamic data along with respective variations obtained from literature or estimated using *in silico* and *in vitro* approaches. Model parameters were randomly varied using Monte Carlo simulations to reproduce the variability in toxicokinetic and toxicodynamic profiles in humans acutely exposed to DZN. This enabled the calculation of a chemical-specific adjustment factor (CSAF) accounting for interindividual differences in both toxicokinetics and toxicodynamics when establishing an acute reference dose. In addition, the model predicted DZN dose-dependent distributions of erythrocyte AChE inhibition for average and sensitive individuals, from which a point of departure (POD) incorporating inter-individual variations was derived via benchmark dose (BMD) analysis for risk assessment. Results of the current study indicate an adequate model performance through comparisons of model predictions with *in vivo* toxicokinetic and toxicity data reported in human volunteer studies. With the validated model and BMD analysis, POD values for the average (the median) and the sensitive (the 99th percentile) subgroups within the general adult population are derived, showing an 8-fold difference due to especially toxicokinetic and not toxicodynamic variability between individuals. The 99th POD prediction (0.25 mg/kg body weight) is comparable with the currently reported no-observed-adverse-effect level (NOAEL, 0.2 mg/kg body weight) for acute oral DZN exposure derived from human volunteer studies. The calculated CSAF at the NOAEL is lower than the default uncertainty factor of 10 accounting for human variability in toxicokinetics and toxicodynamics in risk assessment. Altogether, this study offers mechanistic insights into the interindividual variability in OP-induced AChE inhibition and also provides a strategy for applying non-animal methods for chemical safety assessment.

Presentation: Oral

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Overcoming resource constraints in NAM validation: Strategies for securing funding and ensuring success

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The validation of New Approach Methodologies (NAMs) for chemical safety testing faces significant resource constraints, necessitating innovative strategies for funding and implementation. The EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL), comprising 33 specialized laboratories, supports the European Commission's efforts to validate alternatives to animal testing. An OECD workshop in December 2023 addressed operational and financial aspects of methods validation, emphasizing the need for multi-source funding and good practices for integrity and transparency [1]. Key strategies to overcome resource constraints include: 1) multi-stakeholder funding; 2) prioritization based on regulatory relevance; 3) test readiness criteria; 4) collaborative efforts; 5) awareness raising among funding bodies.

The European Commission is preparing a "Roadmap towards phasing out animal testing for chemical safety assessments," [2] scheduled for publication in Q1 2026, which will address funding for prioritized methods. Additionally, a policy conference organized by the Dutch Ministry of Infrastructure and Water Management in early 2025 focused on developing a European Test Method and Validation Strategy, addressing challenges in continuity, funding, and prioritization.

Overcoming resource constraints in NAM validation requires a multi-faceted approach involving diverse stakeholders, strategic prioritization, and innovative funding mechanisms. Implementing these strategies and leveraging initiatives like the EU roadmap will accelerate the transition towards more efficient, cost-effective, and ethically sound approaches to chemical risk assessment.

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Presentation: Oral



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Manufacturers' perspective on the implementation of non-animal testing for vaccine batch release

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The search for alternatives to substitute the use of animals in the development of analytical methods for Quality Control (QC) of vaccines has been a growing priority on the global scene. It is estimated that up to 10 million animals are used annually, mainly for batch-release testing of vaccines and other biological products. This high number raises, in addition to ethical concerns, important scientific issues, such as variability in *in vivo* responses, which can affect the consistency and reliability of results. These tests, often invasive and painful, can present high levels of variation, which results in delays in the release of batches of vaccines and increased costs. In response to these concerns, the movement towards implementing alternatives animal testing has gained momentum, especially in the context of the 3Rs principles (replacement, reduction, and refinement). The focus has been on replacing traditional tests involving animals with more advanced and robust *in vitro* methods that can reflect human biology more accurately. In this sense, the adoption of *in vitro* tests has become the main strategy to ensure the quality of vaccines ethically and efficiently.

An example of this transition that we've been working is the dTpa vaccine (diphtheria, tetanus, and acellular pertussis). Traditionally, the potency test of this vaccine relies on the use of animal models to evaluate the immune response. However, one of the major challenges is reproducing the immune responses observed in living organisms in a laboratory environment, given the biological variation between individuals, even within the same species. The search for alternative methods to evaluate the potency of vaccines, such as the dTpa vaccine, has focused on the development of more accurate *in vitro* tests, using tests of the vaccine's antigenic content. However, the transition to these tests is not trivial, since scientific and regulatory validation still needs to be established globally, and the lack of standardization for these methods is an obstacle to be overcome.

The implementation of alternatives to animal testing faces considerable challenges. The main difficulty lies in the lack of a direct and robust correlation between *in vitro* tests and *in vivo* responses. The complexity of living organisms makes accurately simulating their biological responses in the laboratory a challenging process. To overcome this, continuous and rigorous development of alternative methods is required, which can be validated and accepted by regulatory authorities before their widespread adoption.

These advances represent a significant transformation in the field of Quality Control of vaccines and biological products. The transition to alternative methods not only meets a growing ethical demand but can also improve the accuracy, efficiency, and reproducibility of tests. Collaboration between regulators, manufacturers, and researchers will be crucial for the validation and implementation of these new methods, to ensure

the safety and efficacy of vaccines without relying on the use of animals.

The evolution of alternative methods, therefore, is not only a response to ethical and regulatory demands but also an opportunity to advance vaccine science in a more efficient and humane manner. As these alternative tests are developed, scientific and regulatory validation will be essential to ensure their widespread acceptance, transforming the QA landscape and promoting a paradigm shift in vaccine testing practices.

Presentation: Oral

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Strengthening networks for regulatory acceleration of NAMs: The RENAMA and PReMASUR cases

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The National Institute of Metrology, Quality, and Technology (Inmetro) is a unique institution due to its broad scope of activities in metrology, conformity assessment – including accreditation and its role as a Good Laboratory Practice (GLP) authority – and consumer product regulation. Since 2012, Inmetro has served as one of the three Central Laboratories of the National Network of Alternative Methods (Renama), a key initiative established by the Ministry of Science, Technology, and Innovation (MCTI) to promote the implementation, dissemination, and validation of alternative methods to animal experimentation. The network currently comprises 39 members from academia and the private sector. In 2015, MERCOSUR's regional platform for alternative methods to animal experimentation (PReMASUR) was approved under the Specialized Meeting on Science and Technology (RECYT) and has been coordinated by Inmetro ever since. Another key initiative led by MCTI, PReMASUR leverages Renama's expertise to conduct training activities primarily in Brazil while also providing opportunities for researchers from MERCOSUR countries. To date, PReMASUR has organized 27 courses focused on *in vitro* methodologies, training 299 professionals from Brazil and other MERCOSUR member states. Additionally, Inmetro has conducted four interlaboratory comparisons involving 17 participants, focusing on OECD-validated *in vitro* methods. Our findings strongly indicate that (i) training and dissemination efforts are crucial for fostering the adoption of NAMs and driving change among national GLP service providers, and (ii) periodic independent evaluations of laboratory performance can significantly enhance result quality, even though such evaluations are not mandatory under the Good Laboratory Practice (GLP) management system. This presentation will highlight key advancements and challenges in building capacity for NAMs, particularly in Brazil, to support the regulatory acceptance of these methods while fostering the implementation of Next-Generation Risk Assessment (NGRA).

Presentation: Oral



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Data-driven design of cell-based reproductive toxicity assays

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Within the framework of the NC3Rs CRACK IT Challenge “SAFE”, we aim to develop reliable *in vitro* bioassays that can predict chemical impact on fish reproduction. To achieve this goal, we developed a computational pipeline to identify novel biomarkers of reproductive toxicity and test their expression in permanent rainbow trout (*Oncorhynchus mykiss*) cells.

The biomarker identification pipeline consists of three components. Firstly, we performed a systematic search of scientific literature and AOPWiki and converted this information into a computable format. To help interpret the accumulated knowledge, we constructed an interactive AOP network and used the topology of the network to identify candidate biomarkers. Secondly, we constructed a Protein-Protein Interaction (PPI) network using the STRING and KEGG databases. We used nodes from the AOP network as seed nodes in the PPI network and collected proteins closely interacting with the seed nodes. Lastly, we constructed a gene expression database by curating and combining transcriptomic datasets that reported reproductive adverse outcomes in fish. We then collected the topmost commonly occurring gene names in these studies. With this strategy we identified 372 potential biomarkers that are (1) mechanistically anchored to adverse outcomes, (2) thus far poorly studied, and (3) show consistent regulation in reprotoxic conditions in fish.

In the next step, we measured the expression of these potential biomarkers in rainbow trout cells derived from organs involved in reproduction and readily available to us at the start of the project – liver and testis. To do this, we have exposed the cells to four chemicals with known reproductive effects in fish, i.e., positive controls (17 β -estradiol, 17 β -trenbolone, fadrozole, and flutamide) and four negative controls (n-octanol, saccharin, sodium dodecyl sulphate, and sodium pentachlorophenol) and measured the expression of all 372 biomarkers using the Standard BioTools microfluidic chip platform. We then discarded genes that showed inconsistent regulation as well as non-specific responders. These filtering steps helped us to select 91 promising genes that show regulation by positive controls whilst not being affected by negative controls.

To anchor the newly generated *in vitro* expression to historical *in vivo* data, we collected fish reproductive toxicity data from HESI, US EPA, GEO and Pubmed databases. As these data were originally reported in different formats, units, statistics, etc., we standardized the diverse information and combined them into a unified fish *in vivo* reprotox da-

tabase. We are now using this database to train machine learning (ML) models to accurately predict *in vivo* toxicity from *in vitro* data.

Lastly, we are currently testing the expression of the promising subset of potential 91 biomarkers under exposure to chemicals with probable reproductive effects in fish. We see this as the first challenge to their efficacy as predictors of reproductive toxicity and the first test of the ML models. Going forward, we envisage this work to result in a two-pronged fish reproductive toxicity assay that falls into the animal replacement category: gene expression measurement in a panel of cultured fish cells and data interpretation with a ML algorithm.

Presentation: Oral

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3R alternatives in the education and training of scientists and lab animal personnel

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This presentation will describe the work done by Norecopa and its predecessors to collect and disseminate information about resources that can replace, reduce or refine the use of animals or animal material in education – at all levels, from replacing dissections in schools, through undergraduate teaching in preclinical subjects to courses for scientists and laboratory animal personnel.

Information about these 3R alternatives has been collected in the NORINA database since 1991. NORINA, which currently contains over 3,400 records, is fully integrated into the Norecopa website, which allows seamless searching through a range of other relevant datasets as well, with links to many external resources. Examples of the large range of products available will be given. The presentation will also highlight the initiatives being taken to produce homemade educational and training aids, at a fraction of the cost of commercial products – and will also discuss how and when they can be used.

Implementation of alternatives is dependent upon successful dissemination of information about them. This presentation will therefore also discuss the ways in which this is currently being done, with advice on how to improve the situation.

Attempts to introduce animal alternatives are often met by skepticism, for a variety of reasons. This resistance to replacement will be discussed, along with suggestions for solutions to the issues raised.

Presentation: Oral



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Applicability of generic PBK models to chemicals

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Next generation risk assessment aims to refine, reduce and replace animal testing by the implementation of new approach methods (NAMs). *In-vitro* to *in-vivo* extrapolation (IVIVE) enables *in vitro* cellular measurements to be implemented into physiology based kinetic (PBK) models. These models utilize detailed biological and chemical data to simulate how substances are absorbed, distributed, metabolized, and excreted (ADME) within the system. However, the variability in model structure, assumptions, and mechanisms complicates the comparability and applicability of PBK assessments, particularly for chemicals, as most existing models focus on drugs. This work addresses the need for the applicability assessment of generic bottom-up PBK models that utilize *in vitro*/silico parameters to predict chemical substances.

27 chemicals in 50 published PBK models addressing single oral exposures with sufficient input and validation data were identified. To enable comparisons of PBK publications to an animal-free generic model workflow, a generic PBK model resembling most commonly used structures and assumptions was constructed and run for a variety of chemicals with *in-vivo* human or rat oral exposure data. *In-vitro* measured inputs of a substance's kinetic used in the model workflow are the apparent permeability measured in Caco-2 cells, fraction unbound in plasma (*f_{up}*) and hepatic clearance (*Cl_{int}*).

Input parametrization was based on the concept of new-approach-methodology and relied on animal-free *in-vitro* data. Data gaps, if occurring, were filled by *in silico* predictions. The modelled and measured results were collected and compared with those derived from a generic and simplistic modelling workflow. Performance was assessed by kinetic indicators such as maximum plasma concentration (*C_{max}*), timepoint of *C_{max}* (*T_{max}*) and the area under the plasma concentration curve (*AUC*).

The simple generic PBK model generated fairly precise *C_{max}* and *AUC* estimates for around 50% of the compounds in the dataset, with the discrepancies between the predicted and observed values being less than a factor of 10. About 15% of the compounds were under- and 35% overpredicted. More complex PBK models often showed improved performance when fitted with *in vivo* data, especially when additional biological processes, such as enterohepatic circulation, active transport, or renal reabsorption, were integrated. The potential causes for the observed prediction discrepancies and the perspective on a minimal set of NAM data for future chemical risk assessments are discussed.

Presentation: Oral

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External to internal exposure assessment considering methodologies and data requirements

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Understanding the relationship between external and internal exposure is essential for chemical risk assessment. This presentation will explore methodologies for external to internal exposure assessment, focusing on how external chemical exposure translates into internal concentrations. An example will be provided coming from the food sector, where biomonitoring information in combination with physiologically based kinetic (PBK) modelling, *in vitro* and *in silico* methods were applied as integrated approach. With a deep dive into the OECD Guidance Documents No. 331 on characterization, validation, and reporting of PBK models and relevant OECD Test Guidelines. These approaches help refine dose estimations, reduce reliance on animal studies, and improve risk assessments. Integrating OECD-recommended methodologies strengthens scientific evaluations, supports regulatory compliance internationally, and enhances public health protection.

Presentation: Oral



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Addressing animal methods bias in scientific publishing

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There is a growing consensus among researchers, policymakers, and regulators that advancing the development and use of innovative non-animal research methods is essential for improving biomedical translation and reducing and replacing the use of animals in science. Despite the availability of such methods in many applications, confidence is still being established, and uptake has been slow due to a variety of barriers. This presentation will provide an overview of one significant barrier, animal methods bias: the preference for animal-based methods or lack of adequate expertise to properly evaluate nonanimal methods, which results in unfair assessments of animal-free research and potentially serious career consequences. It will include anecdotal, survey, and bibliometric evidence of animal methods bias in the context of publishing and funding peer review. It will also discuss the formation and ongoing efforts of the Coalition to Illuminate and Address Animal Methods Bias (COLAAB), an international collaboration of researchers and advocates. The COLAAB aims to build concrete evidence of the characteristics and consequences of animal methods bias and to develop and implement solutions for overcoming it. Illuminating and addressing animal methods bias is crucial for reducing barriers for researchers using nonanimal approaches and for advancing the important shift away from animal use in biomedical research and testing.

Presentation: Oral

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Predicting human risk and safety of agrochemicals without vertebrate animal studies

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Demonstrating a modern scientifically sound and robust strategy for human safety and risk that applies appropriate and flexible exposure and effects characterization without chemical-specific vertebrate tests to reliably address risk, uncertainties, and deficiencies in data and its interpretation with equivalent confidence as do the currently accepted test guidelines and meet the regulatory needs of the agencies.

Presentation: Oral



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Transforming the evaluation of agrochemicals (HESI-TEA): A framework for modern safety assessment

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The rapid evolution of global challenges – climate change, population growth, and increased pest pressures – necessitates a shift in how we evaluate agrochemical safety. The current paradigm, rooted in classical toxicology methods, is becoming increasingly inadequate to meet the demands of sustainable agriculture. To address this gap, the Health and Environmental Sciences Institute (HESI) launched the Transforming the Evaluation of Agrochemicals (TEA), aiming to modernize safety assessment approaches.

This initiative brings together an international, multidisciplinary committee composed of experts from academia, government, the private sector, and non-governmental organizations. The committee's primary goal is to establish a flexible, science-based framework that integrates advanced methodologies and data-driven approaches to effectively inform human health and environmental risk assessments.

This presentation will outline the TEA project's vision and objectives, highlighting the development of a conceptual model designed to streamline regulatory decision-making. A key focus is to minimizing unnecessary animal testing through exposure-based risk assessment strategies, while ensuring that new agrochemicals can be evaluated confidently and safely.

The session will also showcase initial findings from global case studies, emphasizing the practical application innovative methods and their potential to transform regulatory science for the 21st century.

Presentation: Oral

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AFSA Master Class in animal-free safety assessment of cosmetics and cosmetic ingredients

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The Animal-Free Safety Assessment (AFSA) Collaboration Master Class in animal-free risk assessment of cosmetics and cosmetic ingredients (chemicals) is an in-depth, free, online course that covers all aspects of performing a human health safety assessment for cosmetics and cosmetic ingredients (chemicals) without the need for new animal testing. The course is designed in nine modules: an introduction on animal-free risk assessment; problem formulation, including developing an aim, hypothesis, and initial literature review; consumer exposure including oral, dermal, respiratory exposure, deterministic and probabilistic modeling, and aggregate exposure; predictive chemistry, which is in two parts – *in silico* tools and read-across – and includes detailed descriptions of the types of data and modeling techniques used; exposure-based waiving for the three major routes of exposure, oral, dermal and respiratory; safety of botanicals (complex mixtures/extracts), including history of safe use; bioactivity assessment including descriptions and examples of various types of *in vitro* methods and how to combine these methods to address the major toxicological information needs; internal exposure (dosimetry) including a description of the data and modeling used in physiologically-based kinetic modeling, *in vitro*-to-*in vivo* extrapolation, and integration into risk assessment; and a module that discussing the process of reaching a risk conclusion with a detailed walk-through of eleven case examples describing different problem formulations (chemicals, products, toxicological endpoints). A separate module discusses the global regulatory landscape for cosmetics and chemicals. AFSA invites all interested stakeholders to register and contribute to a more effective, efficient, and ethical safety paradigm for cosmetics and chemicals. This course is designed to provide any stakeholder engaged in chemical safety assessment familiarity with the tools necessary to perform or assess a cosmetic/chemical assessment without creating new animal data, thus facilitating the long-term uptake of animal-free safety assessment. This presentation will provide an overview of the course, run through how to sign up and provide a brief demonstration of the course.

Presentation: Oral



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Animal research ethics in North Africa: Current status, challenges and future prospects

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Animals are often used in biomedical research and science education. The aim of this study was to assess the use of animals in research across North Africa and similarly, awareness and use of alternative approaches, whether for ethical or scientific purposes. North Africa (Tunisia, Algeria, Egypt, Morocco, Libya and Mauritania) constitutes a strategic geographic link between sub-Saharan Africa and the northern side of the Mediterranean Sea. Researchers in the North African region are generally aware of the need for ethical review prior to animal experimentation to ensure the quality of research and animal welfare. Future development of animal ethics committees must design ethical review to meet the national and local needs of North African countries. With the lack of a strategic plan at national level in each country, individual institutions have to establish local animal ethics committees (AEC) and adopt a bottom-up approach. In this fact, under the auspices of scientific councils and the contribution of local Laboratory Animal Science Associations, Tunisia, Egypt, Algeria and Libya were the countries those took the initiative and established a National or local AECs. In fact, the support of international organizations is creating a dynamic such as ICLAS, WHAO and the collaboration occurred between the different regions in the Africa continent. It is essential that efforts are made to develop common ethical objectives and outcomes to ensure that all stakeholders are aware of developments in laboratory animal science and progress in the application of the Three Rs and animal welfare. It will also become crucial to encourage "alternative" approaches to be used and ultimately, non-animal methods will be used to promote animal-free science in research, testing and education throughout the African region.

Presentation: Oral

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Advancing neural tissue engineering: A long-term 3D bioprinted brain model for Alzheimer's research

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Introduction: Translating preclinical findings to human studies remains a significant challenge in neuroscience, largely due to the lack of physiologically relevant human models.

Objective: Here, we developed the first human induced pluripotent stem cell (hiPSC)-derived 3D-bioprinted brain model capable of sustaining long-term cultures of functional neural and glial cells.

Methods: Neuronal precursor cells (NPCs) were 3D bioprinted with a collagen-based biomaterial (bioink) in a multilayer wood-pile structure designed to mimic human cortical architecture with high speed and spatial resolution. FRESH method – consisting of printing into an agarose support medium – prevented structural collapse, one of the key limitations of current 3D bioprinted brain models.

Results: Under these conditions, a functional 3D neuroglial culture was maintained for up to nine months. Upon differentiation, NPCs matured into functional excitatory and inhibitory cortical neurons, forming synaptic networks that extended across model layers. Astrocytes spontaneously differentiated from NPCs within the first month, while hiPSC-derived microglia were separately differentiated in 2D and subsequently integrated into the 3D culture, creating a fully engineered neural tissue system.

This model was used to investigate amyloidogenesis in Alzheimer's disease (AD). NPCs carrying the familial AD London mutation were bioprinted in an A β fibril-conditioned bioink. When cultured in 3D, disease phenotypes accelerated, with the soluble A β 42/A β 40 ratio dropping within five weeks, mirroring human biomarker changes. Immunostaining confirmed increased accumulation of mOC87- and 4G8-positive A β deposits within 3D-printed samples, while infiltration of hiPSC-derived microglia has shown to enhance A β clearance.

Discussion: In the absence of a vascular system, nutrient and oxygen exchange is limited to passive diffusion within a 500 μ m range. Our optimized bioprinting approach ensures high spatial resolution and prevents hypoxia and necrotic core formation, common limitations in organoid cultures. This enables long-term survival of the 3D model, which is critical for recapitulating time-dependent neurodegenerative mechanisms such as those in AD. Additionally, the incorporation of tunable, compositionally distinct bioinks provides precise experimental control and allows for the spatial compartmentalization of different cell types.

Conclusions: Our model overcomes key limitations of current 3D bioprinted brain models, including structural definition, long-term viability, and functional integration. This 3D bioprinting approach enhances reproducibility and reduces heterogeneity, addressing challenges faced by organoids and neurospheres. Overall, we present an innovative, human-relevant platform for studying neural network formation, cell-cell interactions, and neurodegenerative disease mechanisms. With its long-term stability and physiological relevance, this model holds great promise for drug discovery and personalized medicine without reliance on animal models.

Presentation: Oral



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Development and regulatory acceptance of NAMs in Japan: Current status and future

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Toxicity evaluation of chemical substances has traditionally been carried out using animals, but with the rise of animal welfare, the development of NAMs, which are safety evaluation methods that do not use animals, has progressed. In particular, in Europe, safety evaluation using animals in cosmetics development is prohibited, and this movement is spreading worldwide. In addition, the promotion of the 3Rs is advocated in the European chemical substance management system REACH and the ICH guidelines that aim for international harmonization of pharmaceuticals. For these reasons, the development of NAMs, their administrative use, and international standardization are important issues in Japan as well. JaCVAM was established about 20 years ago with the aim of contributing to the development of regulatory science in Japan by promoting research aimed at the international standardization of Japanese methods for NAMs that evaluate various toxicity, etc., under a cooperative system with other organizations both domestic and overseas, and actively participating in discussions on the state of standardization of NAMs in international administrative organizations such as OECD, and working to ensure that these NAMs can be incorporated into domestic and international administrative guidelines and contribute to the development of pharmaceuticals, cosmetics, etc.

In this presentation, we will introduce NAMs that are being developed in JaCVAM or related activities for each sector, such as cosmetics, pharmaceuticals, and chemicals, and also introduce the status of regulatory acceptance in Japan. For cosmetics, Japan has a quasi-drug application system, and applicants must submit safety evaluations of the target products and ingredients to the authorities. Although many guidances for alternatives have been published for local toxicity such as eye and skin, no guidances have been issued for systemic toxicity such as repeated dose toxicity and reproductive and developmental toxicity. Therefore, a study project has started with experts from industry, regulation, and academia to systematize evaluation methods using read-across and to issue guidance of alternatives.

In addition, many alternative methods for chemicals have been developed in Japan and have been made into guidelines by the OECD. It is particularly noteworthy that nearly half of the test guidelines for skin sensitization were developed in Japan. Furthermore, an OECD test guideline for immunotoxicity have been developed in Japan recently. Currently several research projects are conducting with the aim of developing test guidelines of alternatives for toxicokinetics, reproductive and developmental toxicity, and respiratory sensitization using MPS. For pharmaceuticals and medical devices, reproductive and developmental toxicity test methods using fertilized zebrafish eggs and artificial placenta models have been developed, the MAT method for testing pyrogens has been developed and included in the Japanese Pharmacopoeia, and skin sensitization test methods for medical devices have been developed, etc. However, there are voices saying that these alternatives

have not been enough implemented in Japanese society despite being listed into guidelines or guidance, and there is a strong demand for domestic collaboration between industry, regulation, and academia under international cooperation.

Presentation: Oral

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Development and profiling of fish cell lines for climate change research and environmental toxicology

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Background and innovation: The establishment, characterization, and optimization of cell lines are crucial in basic research, particularly with regard to the 3Rs (Replacement, Reduction, Refinement), as well as in biotechnological applications. Although fish cell lines have mainly been used for toxicological and virological studies, they have not yet been sufficiently researched despite their potential. Especially in basic research, where too many *in vivo* studies are still being carried out, *in vitro* studies should be used. This work provides an overview of the development and characterization of several novel fish cell lines derived from species of both economic and conservation importance. Additionally, we describe their responses to climate change stressors, like temperature increase, as well as their potential for optimization in high-throughput environmental toxicity screenings and advanced biotechnological applications.

Experimental approach: Stem cells were isolated, and cell lines were established from various fish species. We characterized the species-specific properties and requirements, testing their resilience to climate change stressors, and metabolic changes induced by temperature change. The effects were evaluated through cell imaging, real-time label-free impedance monitoring, oxygen consumption and glycolysis assays, as well as gene expression analysis to track key alterations in cell health and behavior. Additionally, toxicological studies were conducted to investigate the potential of these cell lines for screening environmental pollutants – especially in regard to temperature increase.

Results and discussion: The response of the fish cell lines mirrored the biological characteristics of their species of origin, displaying varying degrees of resilience and adaptation to environmental stimuli and pollutants. We demonstrated that their properties remain stable across later passages and after freezing, enabling their propagation, the creation of a biobank, and transfer to other laboratories. Since these cell lines are derived from both pre- and post-hatched whole fish larvae, they retain the stemness of the embryonic tissues from which they originated. These findings suggest that, when optimized with suitable culture technologies, these fish cells could serve as a reliable system for toxicology and climate change research, and their properties could be leveraged for other biotechnological and industrial applications.

Presentation: Oral



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The future of regulatory application of IATA approaches

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When introducing an IATA for a systemic health hazard like carcinogenicity we run into several challenges including the need to cover the relevant biological space through different non-animal methods, but also the expectation to predict the adverse outcome, the formation of a tumor. Looking closer at the first problem, we assume that many intermediate effects are common between different systemic health effects (e.g., carcinogenicity, reproductive toxicity, specific target organ toxicity, etc.). In fact, a chemical must be bioactive to cause such an effect and become systemically available in a sufficient internal dose to cause, or contribute to, the adversity of interest. We have found this is indeed the case when comparing chemicals fulfilling the hazard criteria for different systemic hazard classes in the Globally Harmonised System for classification and labelling for chemicals. Different systemic health effects show large overlaps between classified chemicals. Applying this knowledge, it would be more efficient not to predict the specific adverse effect, but classify for the hazard of any systemic adversity, based on observed intermediate effects, avoiding the need to observe each specific adversity. The concern then becomes whether we are over- or under-predictive. Therefore, we suggest that as a first approach we assume that the current system is protective enough, so without predicting the adverse effect, we can calibrate a new systemic hazard class with already assessed chemicals. This is the challenge in the EPAA Designathon launched in 2023, and now in a second co-creation phase. In this way, we hope to set up a less burdensome system of information requirements and move toward a system not relying on animal testing. Further we could imagine to risk manage chemicals based on the same principle, by starting to define risk categories and by reverse engineering our understanding of already risk assessed chemicals to define the categories, and then categorize non-assessed chemicals based on the new risk categorization approach. Information requirements for complex endpoints will continue to be addressed by using IATA. It is imperative though that these IATA are designed in an appropriate way from the onset and should be judged on how they could be used to ensure an equivalent or better level of protection, rather than on their predictivity of current animal studies.

Presentation: Oral

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Beyond animal testing: State-of-the-art new approach methodologies (NAMs) for local tolerance assessment

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The question of whether complete replacement of animal testing ("1R") is a worldwide reality for ocular irritation assessment of ingredients is complex and requires a nuanced answer. The European Union's ban on animal testing for cosmetics has driven the development and acceptance of alternative methods, creating a strong incentive for 1R replacement. The cosmetic industry has undergone a fundamental transformation, driven by ethical considerations and scientific breakthroughs, to substitute animal testing with advanced New Approach Methodologies (NAMs). This transition emphasizes demonstrating local tolerance (skin and eyes) through innovative *in vitro* and *in silico* approaches, including 3D reconstructed human tissue models, cell-based assays, and computational modeling.

This discussion highlights the progress and adoption of NAMs, focusing on state-of-the-art methods for assessing eye hazard identification and emphasizing their ability to address complexities that traditional animal tests cannot. We will also highlight a data-driven assessment process and the importance of OECD validation and standardization (TG492B, TG 467). The increasing regulatory acceptance of NAMs and the development of Defined Approaches (DA) are helping to replace animal testing, and the trend is strongly toward using NAM and DA in the daily practice of risk assessment. Case study and decision tree for ingredients are presented, reaffirming the transition away from animal testing.

Although significant progress has been made toward "1R" in ocular irritation assessment, challenges in standardization and regulatory harmonization must be addressed to fully achieve this goal across diverse sectors.

Presentation: Oral



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Qualifying MPS-based assays for pharmaceutical toxicity testing and beyond

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Over the past decade, microphysiological systems (MPS, organ-on-chip) used in academia have matured significantly, as evidenced by their widespread application and the ever-increasing number of high-quality research publications. A wide range of single and multi-organ chip systems have been developed to revolutionize basic research in human biology, the development of curative therapies and the precision of personalized medicine. However, widespread industrial adoption of MPS has been slow, despite strong interest. Bridging the gaps between academia, the pharmaceutical and consumer industries, and regulatory agencies remains the key to rapid progress in the field. Stakeholders need to overcome qualification and validation hurdles to gain confidence and acceptance of MPS-derived data, the latter being particularly important in a regulatory environment. The presentation will summarize the status quo of MPS, provide examples of industrial qualification of MPS-based assays, and discuss next steps to support regulatory decision making globally.

Presentation: Oral

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From ideas to synthesis: Bases for systematic reviews in animal research

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Background: Systematic reviews are rigorous and comprehensive literature syntheses, providing a complete overview of a given topic. Since the 1980s, they have been cornerstones of evidence-based medicine and have contributed significantly to many breakthroughs. Systematic reviews use increases in preclinical research, notably for animal and *in vitro* primary studies. Their overviews provide useful information for future research, highlight knowledge gaps, and can even help to prevent the unnecessary use of animals. Yet, many stakeholders struggle to interpret and evaluate outcomes from a systematic review.

Objective: We aim to provide attendees with a better understanding of systematic reviews' best practices, benefits, and limitations to help them interpret and use systematic review data, particularly for the 3Rs.

Content: The structured process behind a systematic review takes an idea and shapes it all the way to the highest level of evidence by combining all data on a given topic. This process consists of identifying, selecting, appraising and synthesizing data using reproducible methods and a

priori defined criteria. Knowing and understanding these steps is key to interpreting and using systematic review data.

This presentation will cover key methodological considerations for conducting systematic reviews of animal studies, from protocol development to data analysis. We will briefly distinguish between different types of reviews and the data types that can be expected from each. Then, we will explore strategies for contextualizing findings within biological frameworks. This segment includes addressing variability in models and experimental conditions and how to use review data to inform future studies (e.g., for power calculations, predictions, and identifying the best model). Moreover, we will use examples to explore how systematic reviews can inform evidence-based decision-making in policy making and the refinement of animal models. Lastly, we will discuss common limitations in systematic reviews and how to assess a review's quality.

Conclusion: As the field grows, a better understanding of systematic reviews' methodology and limitations will be essential for maximizing their impact. By fostering awareness and critical appraisal, researchers will be able to use systematic reviews to conduct reproducible and ethical research with or without new animals.

Presentation: Oral

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Introduction to the revision of OECD Guidance Document 34 on method validation

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The OECD Guidance Document 34 (GD 34; [1]), published in 2005, has been a key resource for validating test methods used in hazard and risk assessment. Recognising the significant advancements in toxicological science since then, the OECD Working Party of National Coordinators of the Test Guidelines Programme (WNT) approved a project in April 2023 to revise GD 34. This revision aims to promote the timely adoption of New Approach Methodologies (NAMs) and offer practical guidance for validating various method types. The project, co-led by the EU (JRC/EURL ECVAM), the United States, and the Netherlands, addresses key considerations identified by the WNT [2,3] and recent publications. A project group was formed, holding its first virtual meeting in December 2023, followed by face-to-face meetings in April 2024 and December 2024. Discussions during these meetings covered method readiness and validation, Defined Approaches (DAs) validation, validation of mechanistic methods, transferability, and reproducibility studies. Subgroups were established to focus on DAs, readiness criteria, and transferability. The project group also worked to increase consensus on templates for readiness criteria, training and transferability issues, the role of prevalidation, reference to protected elements, and the evaluation and clarification of reproducibility in validation studies. In 2025, two online meetings and one face-to-face meeting are planned, underscoring the ongoing commitment to enhancing the validation process for both new and updated test methods in hazard assessment.

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Presentation: Oral

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In vitro development of neonatal epidermis model (Boti Baby Skin®) for safety and efficacy assessment of cosmetics destined for the pediatric public

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Introduction: Fetal skin development continues until the 34th gestational week, reaching significant maturity for extrauterine life, but neonatal skin continues to mature during the first years (Stamatas et al., 2011). Studies show that neonatal skin exhibits increased gene expression related to extracellular matrix formation, while adult skin prioritizes epidermal homeostasis and immune function (Visscher et al., 2021). The neonatal stratum corneum and epidermis are thinner, making the skin more sensitive (Cooke et al., 2018). Given this, infant skin products must be formulated with gentle, safe ingredients to prevent irritation (Rahma and Lane, 2022). *In vitro* models are essential to assess product safety before clinical trials, as they can accurately replicate neonatal skin characteristics (Suhail et al., 2019).

Objective: Development of a reconstructed epidermis model that mimics the morphological characteristics of neonatal skin for evaluating products and ingredients cosmetic designated for children.

Methodology: A comparative study was conducted between the adult reconstructed epidermis model and the developing neonatal epidermis model. The adult model was created using adult keratinocytes over a differentiation period of 10 to 12 days, while the neonatal model used neonatal keratinocytes with a reduced differentiation period of 7 days. Comparative histological analysis (HE staining) was performed, along with gene expression evaluation of genes associated with the stratum corneum (FLG, KRT10, KRT14, CDH1), followed by immunohistochemical analysis of the corresponding proteins. Additionally, the neonatal model underwent skin tolerability testing using cell viability measurement (MTT), evaluating responses to SDS (positive control) and a cosmetic active complex intended for the Baby product portfolio, to assess tissue fragility during cellular differentiation.

Results and discussion: The results indicated that the cellular origin did not influence the model's sensitivity, but the differentiation period was directly related to tissue fragility. Gene expression analysis showed an increase in the expression of genes associated with the stratum corneum between days 7 and 10 for neonatal cells. Immunostaining was performed for qualitative assessment. In the skin tolerability analysis, the epidermis treated with the cosmetic active complex did not affect cell viability, demonstrating the safety of the active ingredient. In contrast, the positive control (SDS) reduced cell viability by 40% in the epidermis treated on days 7 and 8 of differentiation, supporting the notion that the differentiation period is closely related to tissue fragility. As



the next steps, we will further investigate the modulation of these genes in both adult and neonatal skin to better understand the structural differences between the models at the molecular level.

Conclusion: The developed *in vitro* neonatal epidermis model proves crucial for evaluating the safety of cosmetic products for infants, particularly in assessing differences in skin barrier function between adult and neonatal skin.

Presentation: Oral

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Environmental assessment of safe by design products

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In Europe, chemical substances will one day be evaluated under the Safe and sustainable by design (SSbD) framework. This includes 5 levels of evaluation. The first level is hazard assessment (both human health and environmental) and given the “zero pollution” ambitions of the EU, one might consider it the most critical. At this stage, the endpoints outlined in the REACH Annexes (VII to X) are still considered the gold standard, and derogations from the standard methods are outlined in Annex XI. While Read-across and waivers were mostly used a decade ago, New Approach Methodologies (NAMs) have grown in popularity. In particular *in silico* NAMS which can be built faster, are very rapid and relatively cheap to use compared to experimental studies, have a great advantage for reviewing and comparing hazard profiles of potential new substances (e.g., for ecodesign) in batch mode.

SSbD will require long term assessment of hazard properties as acute effects will not be sufficient to compare relative toxicity of substances especially if they are successful on the market. Cases of regrettable green-lighting and regrettable substitution abound in the literature

(DEHP, BPA, PFOS and their proposed replacements). For eco-design, environmental considerations are as important as human health, as ultimately man is exposed via the environment (one health paradigm). Today, critical environmental properties are Persistency, Bioaccumulation, Mobility and Toxicity.

In this presentation we will discuss rapid methods to assess these properties, current limitations, some things to watch out for when applying QSARs (AD issues) and a brief case study. Unlike *in vitro* methods, *in silico* models provide predictions on the same endpoint as *in vivo* studies and may therefore, if of high enough quality, be used to directly replace them. For environment, *in silico* NAMs are constantly improving both in terms of accuracy and also in AD coverage. Nevertheless, for regulatory use, strict evaluations of the model and the prediction are necessary, and the OECD QSAR Assessment Framework (QAF) recently clarified the elements necessary for assessment. The regulatory documentation (and vigilance) level has significantly increased.

Numerous types of models exist (fragmental approaches, simple linear regression, multiple linear regression, KNN or more recently machine learning approaches). For ecotoxicity predictions, models may be statistical or mechanistic. Classical *in silico* methods such as ECOSAR based on specific structures, and VEGA (using a variety of approaches) are available as freeware. Such models do not include the necessary documentation to understand the pertinence of the results. Commercial software may include generation of such documents as part of the service (e.g., iSafeRat®). The iSafeRat® ecotoxicity QSARs are mechanistically based on a Structural alert (MechoA) scheme which predict toxic mechanisms. Each QSAR is linked to a specific MechoA sub-class to quantify the outcome.

To determine Persistency, freeware and commercial models are available with slightly different outcomes in terms of predicted rapidity of biodegradation. Several models will be explored here. For bioaccumulation and mobility assessments, models are also available which provide a reasonable level of accuracy although there is still room for progress in this area.

Overall, *in silico* models are already able to assess the majority of small organic molecules on the market today and they are constantly progressing.

Presentation: Oral



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New *in silico* approach (QSARs) to replace experimental studies related to human health, physico-chemistry and environment

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New Approach Methodologies (NAMs) have increasingly accepted by regulators due to the joint pressures to reduce animal tests and the need to generate more data rapidly as the number of substances on the market relentlessly augments. *In silico* NAMs are particularly appealing due to their very rapid deployment and relative low cost compared to *in vivo* tests. Nevertheless, the results obtained may be inaccurate if the Applicability Domain of the models is not thoroughly investigated by the user. One important element (often overlooked) is the mechanistic domain. New generation Structural Alert systems using chemical-biological matrix interactions can indicate mechanistic impacts increasing accuracy in predictive toxicology. The MechoA (Mechanisms of toxic Action) scheme for organic chemicals with a MW up to about 1000 Da, was created to include both human health and ecotoxicity alerts to promote a common language for toxicologists at the MIE level of an AOP. The scheme comprises 6 easy to recall main Classes with each of these divided into sub-Classes (28 sub-Classes in total). The scheme links toxic mechanisms rather than just structures (although the mechanisms are based on structures coded as SMARTS within SMILES codes) such that different structures may occur in the same MechoA sub-Class. The hypothesis: As the mechanism is ultimately responsible for the observed effect, molar values of different structures in the same sub-class have the same effect. MechoA is now universally available in the OECD QSAR Toolbox and is currently undergoing a significant upgrade, to MechoA+, fruit of a collaborative exercise, due to be released in the OECD TB this year. The MechoA sub-classes are linked to QSARs for both ecotoxicity and mammalian toxicity (with one QSAR per endpoint) to quantify the impacts generated by the alerts.

This presentation will focus on the MechoA scheme itself and two specific QSARs which are directly linked to this scheme via the sub-classes, the OECD 210 chronic fish test predictions and the OECD 429 & 442A/B LLNA Skin sensitization assay predictions.

Different QSAR methodologies have been used for mammalian toxicology and aquatic ecotoxicity to obtain appropriate quantification:

The fish chronic predictions are based on a simple linear regression of internally validated experimental OECD 210 studies related to a specific MechoA sub-Class, using the most sensitive endpoints (survival post hatch and growth) to determine the EC10, versus the single key descriptor: water solubility.

The skin sensitization QSAR is also generated from MechoA. However, it is not based on simple linear regression. First it will detect whether a substance is a likely sensitizer based purely on our knowledge of sensitizing/non-sensitizing MechoA sub-classes. The decision whether a substance is potentially sensitising, or not is strongly

reliant on the MechoA outcome. Reactive (Class 3) and Pro-active (Class 4) and certain indirect enzyme inhibiting (Class 5) substances are typically sensitizing while Membrane destabilizers (Class 1) and enzymatically hydrolyzed substances (Class 2) are not. Additionally, potential for skin absorption and autoxidation are ascertained by separate modules. If no alert is triggered, the substance is considered non-sensitizing. If an alert is triggered, the potency is evaluated by seeking similar structures using fingerprints. An EC3 value may be determined from nearest neighbors. Model statistics will be presented.

Presentation: Oral

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The AFSA collaboration: Transforming practical knowledge and experience into education through cross-discipline collaboration

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Humane World for Animals (previously Humane Society International) coordinates the Animal-Free Safety Assessment (AFSA) non-governmental organization (NGO)-industry collaboration. Through this collaboration, we brought together experts from our partners to co-author a free, online course that covers the concepts, approaches, and scientific tools that allow a safety assessment of cosmetic products or ingredients without the need for new animal testing data. The course divides the safety assessment process into 8 modules that cover discrete but overlapping concepts, plus an additional module on the global regulatory landscape for cosmetics and chemicals. The course took 5 years to complete and involved the expert input of 59 authors from industry, contract research organizations and NGOs. We hired expert consultants where we had gaps, notably in educational design: development and integration of learning objectives, development of quizzes and exams, and building an engaging and interactive online presentation. Creation of this educational resource through this type of collaboration allowed us to have complete freedom to design a fit-for-purpose program, which in this case was to introduce the use of tools that are relatively new to chemical safety decisions and gave us flexibility with respect to length and design of the overall course and individual modules. It also allowed us to tap the deep expertise of our members who have many years of experience in developing and using these tools. Shortcomings of this approach include a lack of educational development experience, accreditation, or existing channels for dissemination. Even though the course is free, easily available and self-paced, it is also quite involved and can take significant time to complete, and therefore creative means of incentivizing participants are necessary. Nevertheless, the future is in animal-free safety science and approaches, and this course provides a valuable free and accessible resource for learning about the use of these tools in chemical safety assessment.

Presentation: Oral



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Overcoming challenges and facilitating access to informal education in toxicology

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Efforts to implement new toxicological test methods and approaches that do not use animals but rather rely on computational predictions or *in vitro* cell-based methods, are increasing around the world. The process of assessing the risks of chemicals and products is transitioning towards an approach that brings together multiple novel tools for and requires a blend of familiar and new concepts. This transition requires education, engagement, and outreach across regions, career levels, industry sectors, and communities to ensure *in vitro* approaches meet the needs of decision-makers and maintain human health and environmental protection.

Providing a consistent level of educational content and satisfying experiences requires creativity, flexibility, and intentionality – and self-education for new platforms and technology. This presentation will discuss available tools, solutions, and lessons learned over the past five years covering specific challenge areas, including ensuring active engagement from attendees, holding successful events across time zones and languages, and address dwindling funding for or inability to travel to in-person events – while achieving training goals.

Presentation: Oral

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Oh wow! How hands-on and experiential elements lead to impactful learning in toxicology

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Who needs hands-on training? Everyone! Training has been established as an essential element in facilitating the transition in toxicology towards non-animal *in vitro* and computational methods and approaches. While training curriculum can and should be targeted to the needs of the audience and how they will ultimately interact with the approaches and data coming from them, hands-on experiences inject interest and excitement into any event and increase engagement, helping to ensure learning. *In vitro*, hands-on experiences require laboratory space, materials, and staff, and can be quite low-throughput in that the number of trainees can be limited by space and resources, or by the location of the training and the ability to obtain the right materials. Hands-on exercises with computational methods only require a computer and internet access but can also require a large number of staff with relevant expertise and experienced trainers to provide an impactful experience. This presentation will share our organization's experiences with offering hands-on trainings around the world, including challenges and lessons learned.

Presentation: Oral



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Validation and qualification network (VQN) public-private partnerships for adoption and implementation of new approach methodologies (NAMs)

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There is an urgent need to accelerate the validation and adoption of new approach methodologies (NAMs) for biomedical, preclinical, and regulatory uses and to streamline regulatory frameworks for NAMs implementation and adoption. Successful adoption and use of NAMs requires that all sectors with appropriate skills and resources work together in innovative and collaborative ways to meet this urgent need. Accordingly, the NIH in collaboration with the Foundation for the NIH (FNIH) is developing a Validation and Qualification Network (VQN) Public-Private Partnership (PPP) involving scientists at multiple levels of government (including funding agencies and regulators), industry, nongovernmental organizations, and academic institutions to catalyze the development, standardization, validation and use of human-based NAMs that will transform the way we do basic, translational, and clinical sciences.

The Validation and Qualification Network (VQN) is one of 3 components of the NIH Complement Animal Research In Experimentation (Complement-ARIE) Program that will accelerate validation and/or regulatory approval and deployment of NAMs for biomedical research. The goal of the VQN is to establish and apply validation or qualification frameworks by working with regulatory and other federal agencies, industry partners, non-profits, and other collaborators. The VQN will establish a process for evaluating NAMs and channeling use cases through the framework in a pre-competitive environment, paving the way for broader adoption and application by industry and biomedical researchers globally. The goals of the VQN include:

- Accelerating deployment and implementation of NAMs in both research and regulatory contexts.
- Developing standardized reporting and common data elements for preclinical, clinical, and safety performance.
- Developing standardized frameworks for NAMs regulatory submissions.

The VQN will be established through a PPP including an initial Design Phase followed by an Implementation Phase. The Design Phase will include a series of workshops with parties in NAMs-related fields to determine governance and the scope and priorities for NAMs validation and qualification efforts. The Implementation Phase will support

the generation of data packages and regulatory submissions consistent with established validation/qualification frameworks. The VQN will collaborate with the other components of the Complement-ARIE consortium including the Technology Development Centers and the NAMs Data Hub and Coordinating Center, creating a pre-competitive collaborative arrangement that will accelerate the development, standardization, validation, and use of human-based NAMs. The VQN will work closely with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as the US federal resource for validation of NAMs, which will be essential to coordinate across multiple federal partners. By working with collaborators aimed at commercial market use and surveillance, the VQN will help to streamline submission of new NAMs or NAMs-related data to regulatory authorities. Complement-ARIE is specifically focused on the development and validation of novel combinatorial NAMs and will build upon existing U.S. and international efforts to ultimately provide more cost-effective, rapid, human-relevant NAMs for drug discovery, chemical safety testing, and wider biomedical research approaches to bring NAMs products to market.

Presentation: Oral

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New approach methods and next generation risk assessment in the EU

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There have been several initiatives developing new approach methods (NAM, *in vitro* or *in silico* methods) in the past. However, regulatory uptake of these methods is still limited, especially for more complex endpoints like systemic toxicity, carcinogenicity or developmental and reproductive toxicity. To accelerate uptake of NAM into a next generation risk assessment (NGRA) it is therefore needed to link regulatory needs and perspectives closer to NAM development.

In the European Partnership for the Assessment of Risks from Chemicals (PARC) the concept of co-design of NAM as well beta testing of NAM has been explored. Co-design means that regulators priorities different endpoints and projects based on regulatory needs and monitor projects closely by frequent peer review. In contrast beta testing refers to use of selected NAMs in combination for regulatory decision making (if allowed by regulation) prior to their full regulatory implementation.

The presentation will illustrate both concepts and show some examples from the EU PARC initiative.

Presentation: Oral



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Empowering the next generation of toxicologists: Designing effective educational programs for new approach methodologies

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The transition away from traditional *in vivo* methods requires education, engagement, and outreach across regions, career levels, industry sectors, and communities to ensure *in vitro* approaches meet the needs of decision-makers and is a crucial step in ensuring implementation of methods and guidelines. We have seen this very clearly for a number of *in vitro* methods, including eye and skin irritation and dermal penetration. The first principle of training is to address the needs and learning objectives of the audience. Whether a scientist is planning to use *in vitro* or *in silico* methods in the laboratory or will be interpreting the data to make product or regulatory decisions, dictates the necessary training content and format. The sector in which the trainee is working may also dictate the relevant chemical space or types of methods that can be used. Education and training can be a balancing act: we are often pressed for time, resources, and attention, and creative strategies can be employed to maximize engagement, attendance, and reach. This presentation will share lessons and recommendations to address the above challenges using real-life examples from our experience, in particular addressing training priorities for regional and state agencies close to home. Addressing knowledge transfer outside Europe and North America can raise additional logistical and capacity-building needs; our experiences working with regulators and laboratories in China will also be shared.

Presentation: Oral

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The role of kinetics in mixture assessment

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The evaluation of mixture toxicity is of high interest but scientifically challenging. Traditionally mixture risk assessment approaches focus on toxicodynamics and conceptually rely on the dose addition concept, target organ toxicity and mode of action. However, recent results suggest to concomitantly also pay more attention to the contribution of toxicokinetics on potential mixture effects.

Therefore, we introduced the concept of Common Kinetic Groups (CKGs), that includes the consideration of ADME properties and grouping chemicals based on their potential as inhibitors, substrates, or inducers of molecular mechanisms (i.e., enzymes).

This presentation shows first results from compiling CKGs, focusing on the most toxicologically relevant CYP- (Cytochrome P450) dependent enzymes, starting with CYP3A4 but also taking into account other CYPs like CYP1A2 to investigate potential inhibitors and/or substrates within mixtures as well as their combined effects.

We conducted a screening of over 200 pesticide active substances utilizing two open-access and one commercial (quantitative/qualitative) structure-activity relationship ((Q)SAR) tools: SwissADME, SuperCYPs Pred, and ADMET Pred, respectively.

In addition, as proof of principal *in vitro* experiments were conducted analyzing the activity of several compounds as CYP3A4 substrates or inhibitors individually and in binary combinations. By elucidating the interplay between toxicokinetics and mixture toxicity, our research contributes to the understanding of chemical interactions and their impact on human health.

Furthermore, employing *in silico* approaches alongside *in vitro* testing can augment the efficiency of screening efforts, potentially reducing the reliance on traditional *in vivo* studies while ensuring reliable and accurate results.

This approach highlights the importance of leveraging computational tools to complement experimental methodologies and advance toxicological sciences, in hopes to replace animal testing.

Presentation: Oral



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Scientific review in research oversight

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In human subject research involving risk of harm to individuals there is sometimes the potential for personal benefit and almost always a reliance on morally robust agreement to participate. Neither justification is typically available in animal subject research, which instead is justified by appealing to human benefit. Such appeal is universal across regulatory and policy guidance that addresses research uses of animals. For example, the International Guiding Principles for Biomedical Research Involving Animals states, “The use of animals in research, education, and testing is an essential component of the advancement of our understanding about human and animal function. This knowledge is important for advancing human and animal health and welfare” (CIOMS/ICLAS 2012). There are important differences between how various policy bodies appeal to benefit to justify the use of animals in research, but the core argument – that animal use is needed for its benefits – is shared across them all.

Drawing on empirical work conducted by my colleagues and myself, I address US guidance on harm-benefit analysis and corresponding questions about scientific assessment by ethics review boards of human and nonhuman animal research. Some benefits are not worth their costs, and benefits alone are never sufficient to justify activities that come with substantial harms. In the case of human subject research, US guidance demands that ethical review “shall determine that ... risks to subjects are minimized” and “are reasonable in relation to anticipated benefits, if any, to subjects, and the importance of the knowledge that may reasonably be expected to result” (Common Rule 2018). Similarly in the European Union, animal research is subject to oversight requirements to balance harms to animals against expected benefits. In the US, however, guidance on harm-benefit analysis for nonhuman animals is less clear.

Without a mission to review the purported value of proposed science, animal research ethical oversight committees place emphasis on review for animal welfare concerns in lieu of a more thoroughgoing harm-benefit analysis. This approach reveals a conundrum for review committees who attempt to “stay in their lane” – i.e., narrowly follow regulatory mandates while also recognizing that the point of their review is to ensure that research is conducted in an ethically sound manner. An assurance that requires assessment of whether the value of the proposed science is worth the “cost” in animal harm. We found that both human and animal subject ethical review committee members trust others in the research enterprise to ensure science is rigorous and socially valuable. Yet some review members were concerned that their purview is not well enough defined to guarantee the outcomes they seek. Stating, for example, “I think probably the biggest weakness is we have not been able to sort out the proper role of the Institutional Animal Care and Use Committee in their work regarding scientific review. There’s still the question of whether it’s appropriate or not... There’s been almost no guidance on how or what is good practice in that area.”

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Mapping the chemical space of respiratory sensitizers – Can we bridge the gaps?

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Chemical respiratory sensitization represents a complex immunological phenomenon with significant occupational health implications, yet fundamental challenges persist in its characterization, impacting our ability to develop robust predictive models. Current epidemiological estimates suggest respiratory sensitization contributes to approximately 16% of adult asthma cases, though methodological inconsistencies and diagnostic heterogeneity likely result in substantial underreporting. The absence of a universally accepted terminology and diagnostic framework complicates both clinical assessment and regulatory approaches to identify respiratory sensitizers, especially since classification of a chemical as a respiratory sensitizer largely depends on occupational and clinical surveillance.

Existing chemoinformatics methodologies reveal critical limitations in predicting respiratory sensitization potential. Our investigation examined two computational models – Toxtree, a structural alert approach originally developed for skin sensitization, and the Centre for Occupational and Environmental Health (COEH) model, which was designed for occupational asthma risk assessment. While both models individually demonstrated overall good balanced accuracy, each model had unique blind spots, so that when both models agreed there was very high overall accuracy. However, the chemical space of known respiratory sensitizers is relatively limited, and data sets are likely skewed by a detection bias for chemicals with known reactivity domains.

Significant methodological constraints remain, including: (1) lack of standardized diagnostic criteria, (2) incomplete understanding of molecular mechanisms, in contrast to the AOP for skin sensitization (3) no established criteria for potency, and (4) unbalanced data sets that are heavily biased towards positive chemicals and are insufficiently diverse to truly map the chemical space of potential chemical sensitizers, which is critical for the development of robust *in silico* models. Further progress in developing predictive models will require larger data sets curated to a high standard and developed to specifically include chemicals known with high confidence to be negative for respiratory sensitization. Finally, it will require a commitment to proactive data gathering in both occupational and consumer settings.

Presentation: Oral



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AOP-based IATAs to stimulate the use of NAMs: Genotoxicity as a case study

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Current strategies for genotoxicity testing encounter several challenges, such as a high frequency of misleading positive results that lead to unnecessary animal testing, limited mechanistic understanding, inadequate incorporation of innovative methods, and a lack of quantitative assessment. Despite significant progress in technology and scientific knowledge, genotoxicity testing protocols have remained largely unchanged for years. To update genotoxicity assessment and integrate new approaches, the development of Integrated Approaches for Testing and Assessment (IATAs) is crucial. These frameworks combine existing knowledge with data from New Approach Methodologies (NAMs) to reduce or eliminate dependence on *in vivo* testing. Genotoxicity is particularly suited for IATA development, as several advanced non-animal testing methods have been introduced in recent years, such as 3D test systems, Prediscreen[®], MultiFlow[®], ToxTracker[®], and transcriptomic-based biomarkers like GENOMARK and TGx-DDI. However, incorporating NAMs into IATAs must be done systematically and with scientific rigor. The Adverse Outcome Pathway (AOP) concept can play a crucial role in this process, as it links molecular-level events to harmful health outcomes, providing a framework for the structured selection of NAMs. In addition, a comprehensive analysis of the variability of genotoxicity test results from established *in vivo* and *in vitro* methods is needed to clarify to what extent different NAM data may correlate with various *in vivo* data.

Within the context of the European Partnership for the Assessment of Risks from Chemicals (PARC; GA: 101057014), a systematic analysis of the variability of genotoxicity results from all established OECD Test Guidelines is in preparation. Moreover, a global AOP network leading to permanent DNA damage has been drafted and several key events (KEs) and key event relationships (KERs) in the network have been further characterized and/or quantified. In addition, an inventory of NAMs has been compiled. By mapping the different NAMs to the KEs within the AOP network and considering the new knowledge on genotoxicity data variability, IATAs are now being designed and evaluated through case studies addressing specific regulatory questions. Two different case studies have been defined: i) a case study aimed at a NAM-based (i.e., using solely *in vitro* genotoxicity tests) Classification and Labelling of mutagenic chemicals, and ii) a case study aimed at evaluating whether conclusions on the mutagenicity of *Alternaria* toxins can be drawn solely from *in vitro* data. The outcome of these case studies will provide more insights into the practical application of IATAs and help regulators in the transition towards Next-Generation Risk Assessment.

Presentation: Oral

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Toward an expansive interpretation of the 3Rs across areas of animal use

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Lauwereyns et al. (2024, Lab Animal) recently sought to establish a common interpretation of the 3Rs principles in animal research. Their work, co-authored by 22 experts, argued that the original definitions of Replacement, Reduction, and Refinement – first introduced by Russell and Burch in 1959 – have become outdated. Given the rise of New Approach Methodologies (NAMs) and the growing influence of open science and large-scale research collaborations, they asserted that the traditional framework no longer provides a coherent or systematic guide for contemporary research practices. Drawing on extensive discussions within a network of over 200 bioethicists and biomedical researchers involved in the COST Action IMPROVE CA21139 initiative, the authors proposed revised definitions of the 3Rs. These updated definitions look ahead to a broader, more integrated application of the principles, aligning them with the One Health approach, which seeks to promote human, animal, and environmental well-being. In this modern interpretation, the 3Rs emphasize a relative rather than absolute reduction in animal use – advocating for steady, pragmatic progress rather than immediate abolition. The overarching goal is to transition away from animal models in biomedical research, while recognizing that, in some cases, collective decision-making may deem them temporarily indispensable for high-value projects. In such instances, researchers should prioritize minimizing the number of animals used and maximizing their quality of care. This pragmatic approach is encapsulated in the slogan “Less Is More.” The zone of convergence principle – prioritizing achievable, step-by-step progress – is highly adaptable to other areas of animal use, including food production and consumption. Applying the 3Rs in this context requires research and innovation in food science, with an emphasis on understanding the health and environmental implications of alternative production methods. A data-driven, empirical approach encourages collaboration, steering clear of adversarial debates and instead fostering constructive discussions that balance human well-being, animal welfare, and sustainability. However, broadening the scope of the 3Rs presents two key challenges. First, expanding their application beyond animal research requires moving beyond a rigid, technocratic interpretation of the original framework. Second, regional differences in legislation and governance – such as those between East Asia and Europe – can create barriers to consistent implementation. These challenges may generate tensions among researchers, educators, policymakers, and the public, necessitating careful analysis and engagement to foster cooperation. This paper offers an initial exploration of these potential points of conflict, with the aim of identifying areas of common interest where collaboration can thrive. Ultimately, the goal is to ensure that the progressive evolution of the 3Rs remains inclusive, leaving no one behind.

Presentation: Oral



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Environome-driven insights for depression policy: The InDepDash platform

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Depression significantly impacts mental health due to its prevalence and socio-economic burden. Recent global challenges, such as the COVID-19 pandemic and climate crisis, have intensified these issues, particularly affecting vulnerable groups.

The European Commission addresses this through its Comprehensive Approach on Mental Health, emphasizing resilience and inclusivity. Our European Commission Joint Research Centre Science for Policy report [1], informed by expert insights from workshops, highlights depression's complex, environome-driven nature, where personal and environmental factors converge. It advocates for One Health Governance [2] and transdisciplinary collaboration [3], focusing on seven key areas: healthcare, education, arts, technology, economics, humanitarian efforts, and urban planning. These areas align with the Commission's framework for comprehensive, evidence-based policies.

Central to this is the Interactive Depression Dashboard (InDepDash), a dynamic hub synthesizing scientific literature on depression. It supports evidence-based policymaking by bridging human, environmental, and socio-economic dimensions, aligning with One Health principles [4,5].

InDepDash employs digital health tools, semantic ontologies [6], and name entity recognition to identify key correlations and causal relationships. It incorporates the SciWalker semantic search engine for comprehensive document mapping and the MAATRICA metric to evaluate methodological diversity across experimental approaches. The MAATRICA metric utilizes three ontologies encompassing *in silico*, *in vitro* and *in vivo* methods, providing a completeness score that enhances our ability to assess depression risk and resilience, informing targeted interventions at individual and societal levels.

This presentation will showcase InDepDash's methodological approach, emphasizing its potential to drive data-driven policies. By fostering cross-sector collaboration and actionable insights, it contributes to a more integrated, system-wide response to depression's growing burden.

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Presentation: Oral



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Sustainable pyrogen testing – Replacing animal-based tests in the QC laboratory

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For over a century, pyrogenicity testing for microbial safety in parenteral pharmaceuticals and implantable medical devices has relied on the rabbit pyrogen test (RPT). Growing global testing demands and ethical imperatives, including commitment to the 3R principles, have driven the adoption of the Bacterial Endotoxins Test (BET), a critical tool for detecting bacterial endotoxins – the most potent pyrogens – in raw materials, in-process samples, pharmaceutical grade water (WFI), and finished drug products.

Traditional BET methods depend on lysate from horseshoe crabs – specifically *Limulus polyphemus* (LAL) and endangered *Tachyplesus species* (TAL). To address sustainability concerns and reduce reliance on animal-derived reagents, recombinant and cell-based animal-free alternatives – such as the Monocyte Activation Test (MAT) and recombinant Factor C (rFC) assays – have been developed and accepted by global pharmacopeias. However, despite regulatory endorsement, widespread adoption remains limited due to uncertainty in method implementation, transferability, and performance comparability. Furthermore, global sustainability initiatives support the transition to recombinant BET testing.

In this presentation we review the suitable methods for replacement of the traditional pyrogen tests with focus on the animal-free BET workflow using Lonza's rFC-based assay, which provides reagent consistency, broad matrix compatibility, and robustness across routine pharmaceutical sample types. The rFC technology was originally developed by National University of Singapore and licensed to Lonza. The first sustainable rFC assay (PyroGene®) has been commercially available for over 20 years and offers a suitable methodology for implementation in pharmaceutical QC laboratories. Recent protocol optimizations have addressed interference challenges associated with complex formulation samples, such as mRNA-lipid nanoparticles. In addition, PyroGene® assay demonstrates high lot-to-lot reproducibility, facilitating seamless transferability to both QC and R&D laboratories. Alternatively, a protocol for a faster time-to-result (20 min with sensitivity of 0.1 EU/mL) is now available and makes it particularly well-suited for high-throughput and time sensitive workflows. Another key advantage is its compatibility with the PyroTec® PRO Robotic System, which enables automated sample handling – minimizing human errors while enhancing reproducibility and overall productivity.

Finally, we will present side-by-side comparison data to demonstrate the equivalence (sensitivity, reproducibility, specificity, and interference) of recombinant and traditional endotoxin and pyrogen test methods for product safety testing across a panel of standard pharmaceuticals, supporting a practical, ethical, and scalable replacement strategy.

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Practical application of a next generation risk assessment (NGRA) approach for developmental and reproductive toxicity (DART)

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Previously we developed a tiered NGRA framework where we showed that our selection of New Approach methodologies (NAMs) for a first tier of *in vitro* biological activity testing has sufficient biological coverage for the key morphogenetic and cellular events of DART. These NAMs comprised of broad screening tools, including a cell stress panel, high throughput transcriptomics and an *in vitro* pharmacological screening battery covering relevant molecular initiating events and key events from adverse outcome pathways (AOPs) for DART, together with two *in vitro* assays which are specifically applicable for investigating toxicity in early embryonic development (ReproTracker® and devTOX quickPredict™) and CALUX assays covering steroidogenesis, estrogen and androgen activity. DART-specific *in silico* predictions and *in vitro* physiologically based kinetic (PBK) modelling complement the NGRA approach of our DART framework. In our case study, we selected 37 benchmark compounds with a variety of different physicochemical properties and commercial uses. We defined 50 high and low risk exposure scenarios based on traditional DART risk assessments from different regulatory authorities to evaluate the performance within the framework. Points of departure (PoDs) were calculated for each NAM and were compared to internal exposure to estimate a bioactivity exposure ratio (BER). By comparing the lowest PoD across all available NAMs to the estimated chemical exposure (plasma C_{max}), a BER was calculated to characterize the risk of the compound at the specific exposure scenario with respect to DART. A conceptual BER of 1 was used as a threshold to differentiate between high/uncertain-risk (BER < 1) and low-risk (BER > 1) exposure scenarios, and protectiveness was evaluated by comparison to traditional risk assessment decisions. BERs were calculated for all 49 exposure scenarios, and 16 out of the 17 human exposure scenarios classified as high-risk for DART were identified as having a BER < 1. This would conceptually indicate high/uncertain risk and could trigger the requirement for additional assessment to understand potential adversity. Most low-risk exposure scenarios (17 out of 27) were identified as having a BER around 1 or higher, conceptually indicating low risk.

Our results indicate that the framework shows promise for forming the basis of a fit-for-purpose protective first-tier NGRA approach for DART risk assessment. In addition to the evaluation of the protectiveness of the framework, we investigated the technical and biological performance of the different components (*in silico*, *in vitro* biological activity). This deepens the understanding of how the NGRA approach can be implemented for DART risk assessment and how the results within a first-tier assessment can be interpreted and used for a higher-risk tier assessment if needed.

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Introduction on crucial role of exposure assessment in NGRA for humans and environment

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The principles of Next Generation Risk Assessment (NGRA), first articulated for the consumer safety of cosmetics, state that NGRA is exposure-led, hypothesis driven safety assessment approach that is designed to prevent harm. The exposure-led nature of NGRA in particular unlocks significant potential for robust and efficient safety decision making without relying on animal tests. This has been demonstrated in many examples whereby consideration of exposure at the early tiers of the safety assessment enables the use of exposure-based waiving using the threshold of (eco)toxicological concern or directs the generation of relevant *in vitro* bioactivity data. Points of departure from these *in vitro* data can be compared with measured or predicted internal exposure values to determine whether effects may occur at relevant exposure levels, in an approach which is protective of health but not necessarily predictive of hazard endpoints. The tiered and iterative nature of NGRA means that additional exposure-related hypotheses may arise as the assessment progresses, allowing for further refinements and increased confidence in the risk assessment outcome. Examples will be given to illustrate how exposure data has helped to realize the benefits of non-animal methods for consumer safety of cosmetics, and how these benefits can be equally applicable to worker and environmental safety and to other sectors, along with some of the challenges that remain.

Presentation: Oral

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MPS to address challenges in chemical risk assessment

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One challenge in implementing new approach methods (NAM) in chemical risk assessment is the difficulty to appropriately address ADME (absorption, distribution, metabolism, excretion) parameters in single cell culture assays. This is especially important when it comes to analyzing interactions between two or more substances on the level of absorption, distribution, metabolism and/or excretion. As mixture toxicity is getting more and more into focus, such interactions are important to be analyzed beyond the regulation of pharmaceuticals (where such analysis is mandatory), i.e., also in the risk assessment of pesticides, industrial chemicals or food contaminants.

To address toxicokinetics and respective interactions *in vitro*, micro-physiological systems (MPS) like organs-on-chip (OoC) represent an important tool. Such systems may combine a gastrointestinal barrier model (e.g., based on Caco2 cells, representing oral absorption) with fluidics (representing distribution) and a liver-like system (e.g., HepaRG cells, simulating metabolism). By using such a system we have analyzed potential interactions of compounds used in pesticides on the level of absorption and metabolism.

First results are promising, however, to make tools useful for risk assessment, work towards transferability and validation of OoC is needed. In the present talk we will present first results but also discuss the outline of a small ring trial that is ongoing with partners to achieve this level of standardization.

Presentation: Oral



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The evolution of non-animal testing in China: Opportunities and challenges under CSAR

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The evolving landscape of animal testing alternatives in China is a critical topic in the quest for global harmonization in risk assessment. This talk will delve into the implementation of the Chinese Cosmetics Supervision and Administration Regulation (CSAR) and its impact on the adoption of non-animal testing methodologies. The speaker will provide an in-depth analysis of where China currently stands in terms of regulatory and commercial opportunities and challenges posed by CSAR. Given China's significant influence on the global market, this perspective is crucial for understanding the broader implications of regulatory changes in the cosmetic sector. The talk will examine the advancements in adopting and applying non-animal testing methods, the challenges that persist, and the potential strategies for achieving a more harmonized approach to risk assessment. These elements are vital for ensuring regulatory convergence, fostering continued innovation, and promoting collaboration in the pursuit of ethical science.

Presentation: Oral

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Achieving a new paradigm of science-based carcinogenicity assessment for chemicals

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The rodent cancer bioassay, currently the standard method for assessing the carcinogenic potential of chemicals, has several limitations, including being time-consuming, requiring large numbers of animals,

and having limited ability to predict human non-genotoxic carcinogenicity. To address these limitations, new approaches are being developed to modernize carcinogenicity assessment.

Within the development of the EU Commission Roadmap, the new paradigm for carcinogenicity assessment presents an excellent case example for regulatory implementation of New Approach Methodologies (NAMs) and implications for translation of novel approaches into implementable solutions aiming at the same or higher level of human protection compared to the current scheme. Multiple initiatives are underway to develop frameworks for a new approach to carcinogenicity assessment, incorporating weight of evidence (WoE)-based methodologies that utilize *in silico*, *in chemico*, *in vitro*, and short-term *in vivo* tests.

To facilitate the development of a harmonized framework for WoE-based carcinogenicity assessment, the European Partnership for Alternative Approaches to Animal Testing (EPAA) has established a working group, including representatives from key initiatives, ensuring synergies and co-creation. The working group aims to identify opportunities for implementing a paradigm shift, with a focus on achieving risk management goals within a realistic timeframe.

A review of ongoing initiatives revealed significant commonalities, with all of them being built upon mechanistic approaches to identify potential carcinogenicity concerns earlier in the process of developing compounds. Moreover, they share a common rationale for using alternative approaches. This led to the development of an overarching assessment framework, comprising various components that experts and stakeholders must consider to identify current and future opportunities that will enable changing the paradigm. A harmonized approach is essential for successful implementation, which will however require addressing policy differences, identifying commonalities, and determining what regulatory changes are needed to implement a new paradigm. Effective communication with government organizations, stakeholders, and the general public is also crucial.

Taking all this into consideration, a step-wise action plan is proposed, outlining short-, medium-, and long-term actions designed to bridge scientific knowledge gaps and implement the necessary measures for a paradigm change. The ultimate goal is to inform and advance the European Commission roadmap for phasing out animal testing, ensuring a more efficient, effective, and humane approach to carcinogenicity assessment.

Presentation: Oral



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Learning through doing in Global North versus Global South contexts

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Experiential learning is an active learning approach, where learners learn both through experience and simultaneously reflecting on these experiences rather than passively receiving information from educators. It enhances the acquisition of competencies (knowledge, skills and behaviors), promotes deeper understanding, and enables learners tackle real-world challenges with confidence and adaptability. Thus, it is an ideal approach for use in the professional education of all those involved in the care and use of research animals.

In this presentation, I will showcase experiential learning activities used in the education and professional education of different stakeholder communities involved in the care and use of research animals. I will highlight the approaches educators should adopt in delivering these activities so learners get the most out of these experiences.

An individual attitude to animals and animal research is influenced by many factors including their lived experiences, culture, religion and society. Educational activities created for one community or culture cannot be directly transferred to another culture or community. I will share how resources and activities created for the Global South can be modified for use in the Global North.

Presentation: Oral

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Puzzles and problem-solving

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Improved education in experimental design has been seen as important for overcoming widespread concerns that experiments were not being rigorously conducted and well designed. In teaching the topic, entertaining with puzzles and problems to solve has been found good for stimulating engagement and understanding. Feedback on sessions using this approach has shown they are well-liked and instructive. Since introducing scenario-based problem-solving group discussions into 3-day courses some 16 years ago free comment feedback has included many requests for more, and only one “Not keen”. In recent courses with feedback responses of 76 to 100%, participants have picked group discussion as the most helpful session of the day on half the days and always placed it in the top three.

The experimental scenario problems can be set so that they only need the information provided in previous lectures or videos to deal with them. This reinforces the content of the earlier material to which they are linked. In five 2- or 3-day courses with such linking run since 2020 participants were asked whether the group discussions helped understanding of the previous talks and 78% responded definitely and 20% partly.

With this approach the tutor has to concentrate on key elements of design. In preparing a problem involving an experimental scenario a tutor needs to state clearly and concisely the purpose of the experiment, the proposed comparisons, the procedure to be used and the outcome measures. Also tutors have to be prepared to discuss potential solutions they have not thought about should the learners suggest them. Although this can be more demanding of tutors than other teaching approaches, it allows learners to participate more actively than when listening to experts. It has provided a step change in understanding which even experienced researchers have described as “eye-opening”. The points newly appreciated apply to both animal-based and non-animal research.

This talk will give examples of the educational material used and the feedback obtained. After it participants will be given a chance to experience some of the problems as learners would, and to discuss the approach.

Presentation: Oral



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Modelling cardiac arrhythmias with human induced pluripotent stem cells

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Congenital long QT syndrome type 1 (LQT1) is a cardiac disorder characterized by ECG prolongation of the QT interval induced by variants in KCNQ1 gene. But, since LQT1 displays incomplete penetrance, modifier genes may influence its pathophysiology. In this context, the identification of both pathogenic variants and modifier genes constitutes a challenge for better understanding this syndrome and earlier identification of subjects at risk. The objectives of the present study were to verify the pathogenicity of the KCNQ1-I588T mutation through *in vitro* LQT1 modeling as well as to elucidate its mechanisms of incomplete penetrance.

Erythroblasts from a LQT1 proband (a 15-year-old symptomatic patient carrying a KCNQ1-I588T mutation and a SCN5A-H558R polymorphism) and a non-symptomatic familial carrier (having the same KCNQ1 mutation) were reprogrammed to iPS, which were characterized and differentiated into cardiomyocytes for LQT1 modeling. To confirm KCNQ1-I588T as a LQT1-causative pathogenic variant, proband LQT1 iPS were corrected for this mutation using CRISPR-Cas9. Also, inserting SCN5A-H558R polymorphism in the carrier iPS allowed the investigation of incomplete penetrance mechanisms. Statistical analyses were carried out by Student's t-test and ANOVA. Both iPS generated presented normal morphology, karyotype, and pluripotent markers and were then efficiently differentiated into cardiomyocytes. Electrophysiological analyses demonstrated a prolonged action potential duration in proband iPS-CM compared to carrier iPSC-CMs (APD90 = 348 ± 50 vs 194 ± 45 msec, $p < 0.0001$). Correction of the KCNQ1 variant in the proband significantly shortened the APD90 (305 ± 58 msec, $p = 0.0003$). Moreover, the introduction of the SCN5A polymorphism, already reported to increase the QT interval in healthy individuals, induced a significant prolongation of the APD90 in the carrier (327 ± 45 msec, $p < 0.0001$). Based on our LQT1 model, KCNQ1-I588T variant classification can be updated to pathogenic according to ACMG criteria. Furthermore, SCN5A-H558R polymorphism promotes a damaging effect, contributing to the long QT phenotype, which reveals the oligogenic nature of LQT1.

Presentation: Oral

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Applications of NAMs for early derisking: Opportunities and challenges

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Advances in artificial intelligence (AI) and high-biological dense assays such as Cell Painting is revolutionizing the fields of small molecule discovery and toxicology. In this presentation, we explore the integration of AI with cell painting data – a morphological profiling method that captures cellular responses to chemical exposure – to enhance toxicity prediction and facilitate *de novo* compound design. By employing machine learning algorithms, we extract complex patterns from high-dimensional cell painting images, enabling more accurate identification of toxicity profiles and biological mechanisms triggered by chemical compounds. Furthermore, as proof of concept we apply AI-guided generative models to design novel compounds with desired biological properties. This approach not only accelerates the identification of safe and effective candidates but also reduces reliance on costly and time-intensive animal testing. Our findings demonstrate that the synergy between AI and cell painting data holds significant promise for early de-risking and chemical design, paving the way for more efficient and data-driven approaches in chemical safety assessment and small molecule discovery.

Presentation: Oral



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Collaborative approaches to build confidence in the application of microphysiological systems (MPS) for drug development and regulated toxicology

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The application of Microphysiological Systems (MPS), also known as organ-on-a-chip technology, has emerged as a transformative tool in drug development and regulatory toxicology testing. MPS offer the potential to model human physiology more accurately than traditional cell cultures or animal models, providing more reliable data for understanding drug efficacy, safety, and toxicity. However, despite the promise of MPS, their widespread adoption in both research and regulatory environments remains hampered by challenges related to standardization, validation, and reproducibility. Collaborations and cross-party frameworks that guide the development, application, and evaluation of MPS are therefore critical to ensuring their effective use and fostering confidence in their reliability for drug development and toxicology testing.

This talk will explore how as an MPS developer we are actively engaging in collaborative activities and cross-party frameworks to accelerate the integration of MPS into regulatory and pharmaceutical workflows. It will highlight current efforts to create standardized protocols and best practices for MPS development, particularly in the context of toxicity testing and drug safety assessment. By emphasizing robust scientific practices, the talk will illustrate how such guidelines can streamline the validation processes, making MPS a more accessible and trusted tool for pharmaceutical companies and regulatory bodies alike.

Several key collaborative activities will be discussed, including work with the North American 3Rs collaborative and the Critical Path Institute to develop a standardized framework to evaluate MPS tools for assessing drug-induced liver injury (DILI) and other major toxicology assays. The talk will highlight the opportunities and challenges of working alongside a range of diverse stakeholders (inc. regulatory agencies such as FDA) and the key areas of support required for long-term success. Additionally, the talk will highlight several real-world case studies where collaborative approaches with pharma, biotech and CROs have been successful in building confidence in MPS capabilities enabling them to be adopted for a range of applications, leading to faster drug development timelines, more accurate preclinical assessments, and a reduced need for animal testing, all of which are essential to the future of drug safety.

Presentation: Oral

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Industry experience building NGRA case examples to induce regulatory acceptance

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New approach methodologies – NAMs – have been used as replacement for animal testing for local toxicity for decades.

Challenges remain when it comes to addressing mechanisms or endpoints related to systemic toxicity. In the literature, several case studies are published using new approach methodologies – NAMs – illustrating how they could be integrated using frameworks for decision-making.

Common features of the frameworks used to integrate NAMs are their tiered and iterative nature, they help structure the data used in the assessment. They offer flexibility in the methods chosen for the assessment and call for qualifying the level of confidence related to the data.

Despite the examples in the literature, there are very limited practical applications using NAMs to assess systemic related issues in regulatory dossiers; setting aside read-across. The use of NAMs for complex endpoints seems to be challenged for a number of reasons, a few are named here:

- The limited knowledge on the multiplicity of mechanisms as well as the sequence of events that could lead to systemic effects
- Lack of practical guidance on the rationale for choosing the methods and their integration for decision making
- Lack of practical guidance on how uncertainty could be assessed

Case studies run by the Cosmetic Europe long range science strategy using the SEURAT1 framework and following the ICCR principles will be used to illustrate how NAMs can be integrated for decision-making and the related challenges.

Presentation: Oral



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Introduction to IATA in regulatory application

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Integrated Approaches to Testing and Assessment (IATA) is a framework for integrating new and existing data within the context of exposure and use for optimally addressing health concerns. The Organization for Economic Cooperation and Development (OECD) has developed guidance and case examples for the use of IATA in chemical safety assessment. Regulatory acceptance of IATA approaches has thus far been successful mainly for acute endpoints where the methods are designed to replicate adverse effects seen in animals (including humans). More complex toxicities resulting from longer-term, systemic exposure require a different approach to assessing and applying bioactivity. Next Generation Risk Assessment (NGRA) is an exposure-led IATA approach for addressing human health concerns for cosmetics and ingredients without new animal testing. Several NGRA case studies developed by Cosmetics Europe and others have been reviewed and published by the OECD and demonstrate that NGRA can provide equivalent or better information, including quantitative information, on acute local toxicities for safety assessment of ingredients and products with broad human exposure. However, there is a difference between what NGRA approaches provide – identification of safe levels of exposure – and what traditional animal-based studies provide – prediction of exposure that causes a specific effect. There also remain scientific gaps in coverage of NGRA approaches currently provide and the spectrum of toxicological concerns and there is a gap between what some regulations require (e.g., classification for specific systemic hazard endpoints) and what IATA optimally provide (determining levels of safe exposure). Several projects are aimed at addressing these gaps, including the ASPIS Cluster (funded through the EU Horizon 2020 Research and Innovation program) and continued development of IATA case the International Collaboration of Cosmetics Safety and others. Envisioning new ways to apply IATA approaches to regulatory decisions include an initiative from the European Joint Research Center to reframe Classification and Labeling to improve efficient regulation of large chemical inventories. Through these and other initiatives, the barriers to regulatory adoption of IATA approaches will eventually be surmounted.

Presentation: Oral

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Advancing next-generation risk assessment for fragrance materials

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The Research Institute for Fragrance Materials (RIFM) employs a comprehensive, science-driven approach to evaluating the safety of fragrance ingredients, ensuring their responsible use in consumer and commercial products. This session will explore the foundational framework of next-generation risk assessment (NGRA), integrating principles outlined in Criteria for the Research Institute for Fragrance Materials, Inc. (RIFM) Safety Evaluation Process for Fragrance Ingredients and The RIFM Approach to Evaluating Natural Complex Substances (NCS). Attendees will gain insight into RIFM's robust methodology, which systematically assesses human health and environmental exposure while incorporating the latest scientific advancements, including read-across strategies and exposure-based waiving approaches. The discussion will also address the critical role of New Approach Methodologies (NAMs) in refining the safety assessment process. This session is ideal for both new and experienced safety assessors, providing an overview of RIFM's evolving strategy to ensure global fragrance safety.

Presentation: Oral



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Environmental risk assessment of fragrance materials – Expanding region-specific framework

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RIFM has led the scientific advancement of environmental risk assessments for fragrance materials for over two decades, ensuring their safe use in products that may enter aquatic environments. This session will explore the latest updates to RIFM's environmental framework, which applies region-specific (Europe, North America, South America, Asia-Pacific) exposure data and advanced predictive tools to assess ecological hazards and environmental fate. The discussion will include enhancements to wastewater treatment plant fugacity models, integrating abiotic and biotic degradation mechanisms, and applying ecological thresholds of concern. Additionally, this presentation will highlight the importance of the 3Rs (Replacement, Reduction, and Refinement) in advancing sustainable risk assessment practices. By leveraging data-driven approaches and cutting-edge modeling techniques, RIFM continues to refine its tiered strategy for prioritizing fragrance materials, ensuring science-based environmental safety decisions across diverse geographic regions.

Presentation: Oral

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Sentience as the foundation for new legislative and regulatory efforts

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This presentation will begin with an exploration of political and legal philosophy related to the concept of sentience, highlighting the perspectives of key philosophers and ethicists. Following will be a discussion on how the failure to fully consider animal sentience has contributed to systems that routinely prioritize convenience, financial profit, or scientific freedom over the interests and welfare of animals. The presentation will argue that although U.S. law is grounded in the principles of the Three Rs, there is no legal mandate to implement any of them. An argument that laws or regulations governing the welfare animals used in research should extend beyond their time in the laboratory will also be discussed.

The second half of the presentation will provide an overview of recent or ongoing legislative or policy initiatives aimed at reducing or replacing animal use in research and testing, assessing their potential impact or unintended consequences through the lens of sentience. The presentation will conclude with a series of questions designed to foster open discussion and dialogue with attendees.

Presentation: Oral



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Putting experimental rigor at the forefront of ethical review

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This presentation will help ethical review body (ERB) members (e.g., in IACUCs or AWERBs) support the reduction “R” of animal use. The focus will be on encouraging rigorous experimental design, maximizing the chances that animal experiments deliver reliable results and minimizing the risk of wasting animals from poorly designed studies. The presentation will show how outputs from the NC3Rs’ Experimental Design Assistant (EDA), a free-to-use online tool to guide researchers through experimental design, can help identify missing information or highlight where the experimental design could be improved. It will also include how the ARRIVE study plan, a template to ensure procedural plans and experimental design are comprehensively considered, can help ethical review bodies assess the rigor of the research and the refinement of the procedures. These resources together can facilitate the ethical review body process, providing guidance and support to ERB members on recognizing and supporting research best practice. Scenarios, using both good and bad examples, will be used to highlight different ways experimental design might be described in project or study plans and demonstrate how ERB members might respond to these scenarios.

Presentation: Oral

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Advancements of *in vitro* NAMs for regulatory applications: Challenges and opportunities

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Despite the progresses of *in vitro* New Approach Methodologies (NAMs), their regulatory use and the transition towards Next Generation Risk Assessment is still facing major issues, such as, lack of confidence, limited reproducibility and transferability, which hinder their regulatory acceptance. *In vitro* NAMs are challenging to standardize

due to the level of complexity of the *in vitro* models, including integration of immune system, exposure, especially for Air-Liquid-Interface system, dose-metrics, etc. To increase the regulatory acceptance of *in vitro* methods, it is essential that such approaches are further developed and validated. To address these issues, multiple European initiatives – including the HEU projects CHIASMA - GA N°101137613, SCENARIOS - GA N°101037509, MACRAME² - GA N°101092686, PHOENIX - GA N° 953110, and POTENTIAL - GA N°101092901 and the EFSA project TULI (OC/EFSA/IDATA/2023/02) – are actively working to develop technical solutions and increase the relevance of *in vitro* NAMs. These efforts include expanding the biological readouts for regulatory relevant endpoints, optimizing exposure systems, improving data comparability, and enhancing model reproducibility and transferability.

The combined efforts of academia, regulatory agencies, and industry stakeholders focus on tackling the issue from different angles. One critical area involves demonstrating the applicability of *in vitro* methods for regulatory relevant endpoints, such as genotoxicity. To this end, work is underway to demonstrate the extent to which NAMs can be used to predict genotoxicity (e.g., by using COMET or the γ -H2AX assay). The study includes different NAMs, heterogenous chemical classes, and exposure conditions.

Another area of development concerns the generation, use, and regulatory application of NAMs-derived -OMICS data. Within the different projects (e.g., CHIASMA, MACRAME², TULI) different initiatives are ongoing to generate multi-OMICS data (e.g., including transcriptomics, metabolomics, and epigenomics data) and develop analytical pipelines allowing their use in a regulatory framework. Methodological studies on bulk and single cells transcriptomics are being carried over to understand the pro and cons of each technique and, importantly, the reproducibility of -OMICS data when applied to NAMs. These studies will also deepen the mechanistic understating of the biological effects of different classes of chemicals (e.g., PFAS, pesticides, sensitizers) and materials (e.g., nano-pesticides, 2D materials, fibers). This work will also facilitate better definition of the applicability domains of NAMs and inform on their suitability for different regulatory contexts. Moreover, several studies are directed towards the optimization of chemical/material exposure to better reflect realistic inhalation scenarios. This includes comparison submerged exposure, semi-Air-Liquid Interface (semi-ALI), and fully ALI conditions using a range of exposure devices for both liquid aerosols and dry particulates. Activities include the development of standardized templates (e.g., ToxTemp) and structured data reporting templates. Beyond methodological improvements, the physiological relevance of *in vitro* inhalation models is enhanced through the integration of advanced features, such as their integration in multi-organ dynamic systems and the further transition towards serum-free reagents.

Presentation: Oral



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Decoding depression: Exploring the environome across life course. Securing mental health systems: Preparedness, innovation, and policy for global resilience

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Depression, exacerbated by global crises such as pandemics and climate change, remains a critical public health issue, particularly affecting young people and vulnerable populations. In response, the European Commission's 2024-2029 agenda prioritizes mental health, integrating it into socio-economic strategies to enhance resilience [1]. This science for health policy work draws on global expertise and lived experiences, utilizing a transdisciplinary approach to explore depression within the environome framework, which examines the interplay of personal, social, and natural environments [2]. The Interactive Depression Dashboard (InDepDash) supports evidence-based policymaking through real-time data analytics.

Advocating for One Mental Health Governance [3], the findings emphasize comprehensive interventions in crisis preparedness, education, and inclusive research [4-6]. The study highlights the Adverse Outcome Pathway (AOP) framework [7], identifying key adverse outcomes, including the extreme risk of suicide [8], linked to depression. Key factors such as environmental stressors, socioeconomic influences, and genetic predispositions are examined, underscoring the necessity for adaptive policy responses.

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Presentation: Oral



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European Commission Joint Research Centre's transdisciplinary efforts to advance the One Health approach

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The European Commission Joint Research Centre (JRC) is promoting transdisciplinary efforts to advance the One Health approach, addressing global health challenges with innovative strategies. The need to assess chemical effects [1,2] has led to New Approach Methodologies (NAMs) such as *in vitro*, *in silico*, and *in chemico* models, though regulatory acceptance remains challenging. This presentation outlines a framework [3] for quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) to support safety decisions. A unified One Health strategy [4] integrates human, animal, and environmental health. The JRC enhances global health security by addressing the COVID-19 pandemic [5-7], vector-borne [8], and zoonotic diseases [9], using epidemic [10] and wastewater surveillance [11], antimicrobial resistance [12], and non-communicable diseases. Emphasizing mental health, particularly depression, the JRC highlights the “environome’s” impact on well-being [13] and aims to shape resilient global health policies.

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Presentation: Oral



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NGO-sponsored informal educational events accelerate nonanimal science and policy

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Increasingly, scientists and policymakers recognize the benefits of studying human health and disease, and testing products, using methods that reliably translate to humans because they are based on human information. Lack of familiarity and expertise in nonanimal approaches remain challenges for scientists at all career stages. Informal education, including summer schools, webinars, targeted roundtables, and other focused events help scientists build expertise and interest in doing things differently. Dedicated advocates at NGOs offer some of the most creative and accessible options by understanding challenges, filling gaps, and connecting scientists with policy solutions. This presentation will cover select NGO-sponsored informal educational offerings that have led to policy change and adoption of nonanimal methods.

Presentation: Oral

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Advancing organ-on-chip research: A decade of European Commission investment and emerging trends

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Over the past twelve years, the European Commission has significantly advanced the Organ-on-Chip (OoC) technology through sustained funding across multiple Research and Innovation Framework Programmes. This strategic investment of around €300 million allocated to over 100 projects has not only fostered the development of sophisticated organ models but also propelled the integration of these models into regulatory and clinical settings.

Funding overview: FP7 (2007-2013) initiated support to nascent OoC technologies, funding 3 projects with a total budget of €3,8 million. These early projects focused on foundational aspects, such as organ modelling, microfluidic systems and integrated bioelectronics.

Horizon 2020 (2014-2020) marked a substantial increase in support, allocating €151 million to 45 projects. This phase emphasized an increased focus on regulatory implementation and real-world applications.

Horizon Europe (2021-2027) continues this trajectory with €142.1 million dedicated to 52 projects. Current efforts are directed towards further enhancing predictive modelling, utilizing patient-derived tissues and advancing personalized medicine approaches.

Evolution of OoC models: A detailed analysis of these projects reveals a clear progression in model complexity.

Single-organ models (61 projects) serve as the foundation for organ-specific disease modelling. Applications range from investigating tumor-host interactions in liver cancer, accelerating drug repurposing for rare neurological disorders, and improving nanoparticle-based drug delivery across the blood-brain barrier.

Two-organ models (16 projects) enable the study of dynamic interactions between interconnected systems, for instance the gut-brain axis, providing insights into disease mechanisms and therapeutic interventions.

Three-organ models (4 projects) facilitate comprehensive studies on drug toxicity testing, tumor-immune interactions in metastasis, and treatments of cardiovascular and neurological diseases.

Multi-organ models (a select group of projects) aim at improving toxicology testing, developing real-time monitoring and sensing and enhancing regenerative medicine and tissue engineering.

Among the modelled organs, the brain stands out as the most frequently studied organ. Projects target a spectrum of neurological disorders, such as Alzheimer's disease, schizophrenia, brain metastases and neurovascular interactions.

The trend is moving toward the development of multi-organ and whole-body systems, aiming to create more physiologically relevant models for disease study and drug testing. This evolution aligns with the goals of personalized medicine, where models can be tailored to individual patient profiles, facilitating precision drug development and targeted therapeutic strategies.

Presentation: Oral



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EUROTOX early career forum: Empowering the next generation of toxicologists

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The EUROTOX Early Career Forum (ECF) is a dynamic network dedicated to supporting and connecting Early-Career Researchers (ECRs) in toxicology. Committed to fostering the next generation of toxicologists, ECF provides a platform for career development, mentorship, and scientific exchange while promoting the Intersectoral, International, and Interdisciplinary (3I's) nature of science. As an inclusive initiative, ECF welcomes all ECRs, regardless of background, nationality, or identity, cultivating a diverse and collaborative scientific community. Our initiatives are shaped by feedback from the ECR community, ensuring that our activities remain aligned with the evolving needs of early-career toxicologists.

ECF is led by a team of eight ECRs from diverse professional backgrounds – including academia, industry, and regulatory agencies – ensuring balanced representation across nationalities and expertise. While operating autonomously, ECF closely collaborates with the EUROTOX Executive Committee and Subcommittees to align its initiatives with broader EUROTOX objectives. This cooperation strengthens the EUROTOX experience for ECRs by expanding educational opportunities and communication activities, developing European Registered Toxicologist (ERT) informational materials, supporting career exchanges through the EUROTOX Early Career Exchange Program (ECEP), and advancing other career-focused initiatives. Additionally, ECF actively engages in discussions on how to improve training and professional development pathways for young toxicologists, ensuring their voices are heard within EUROTOX decision-making processes.

At EUROTOX congresses, ECF offers a range of tailored activities designed to enhance networking and professional growth. Facilitated networking sessions, sightseeing tours, and poster walkaround tours provide structured opportunities for ECRs to connect, present research in small groups, and receive mentorship from senior EUROTOX members. Science popularization activities such as “Pub Up Your Poison”

event further encourage science communication in an engaging and accessible format, fostering confidence in public speaking and outreach. Beyond conferences, ECF extends its impact through online initiatives, including interactive talk shows that blend scientific discussion with personal insights, featuring engaging moderators and expert guests. Regular blog posts offer congress highlights, toxicology advancements, and insights into ECF activities, strengthening connections within the ECR community.

Furthermore, ECF actively fosters collaboration with other early-career networks, building a broader community that supports the professional and scientific growth of young toxicologists across different sectors and disciplines. By uniting these networks, ECF contributes to a stronger, more connected toxicology community that is better equipped to drive scientific innovation and regulatory advancements. Through these initiatives, ECF equips ECRs with the skills, knowledge, and professional networks necessary to shape the future of the field. With a strong focus on inclusivity, scientific exchange, and career development, ECF fosters a thriving community where ECRs can grow, collaborate, and lead the transition toward innovative and sustainable toxicology practices.

Presentation: Oral

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The maximum acceptable concentration process and the role of quantitative risk assessment 2

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A fundamental aspect of fragrance safety assessment is establishing Maximum Acceptable Concentration (MAC) values for individual ingredients to ensure their safe use across diverse applications. This session will detail the process of determining MAC values within RIFM's safety framework, highlighting how toxicological data, exposure modeling, and risk assessment principles converge to define acceptable usage levels. The discussion will also explore the evolution of Quantitative Risk Assessment 2 (QRA2), a refined methodology designed to improve risk prediction for dermal sensitization. QRA2 employs a tiered approach to assessing fragrance-induced skin sensitization, leveraging updated scientific models to support industry-wide safety standards. Attendees will gain insight into how MAC derivation and QRA2 contribute to ensuring consumer safety and how these approaches can facilitate the integration of future New Approach Methodologies (NAMs).

Presentation: Oral



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Introduction to and history of GIVIMP

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The OECD Guidance Document on Good *In vitro* Method Practices (GIVIMP), coordinated by the European Commission Joint Research Centre's EURL ECVAM, provides a harmonized framework to enhance the quality of *in vitro* methods [1]. Building on foundational practices in cell culture and laboratory standards [2,3], GIVIMP supports the rigorous and reproducible evaluation of chemical safety [4,5]. It outlines ten critical aspects of *in vitro* work, including quality considerations and SOPs, to improve scientific robustness [6-8].

The PathoSeq-QC project extends GIVIMP principles to genomic surveillance [10]. Additionally, MAATrica, inspired by GIVIMP, offers a novel metric for assessing methodological coherence in research papers [9]. MAATrica uses advanced tools to automate knowledge extraction and has been validated on numerous papers [9].

The harmonization of global policies for microphysiological systems highlights the shift towards non-animal methodologies [11]. Advances in animal models and research alternatives further demonstrate the evolution of regulatory science [12].

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Presentation: Oral



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Global harmonization of reconstructed human tissue production: EPISKIN's quality system

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Reconstructed human tissues (RhTs) are valuable *in vitro* tools for safety and efficacy evaluation, offering ethical and scientifically relevant alternatives to animal models. EPISKIN has four decades of experience developing high-quality RhTs for research and testing worldwide. To ensure data integrity and reproducibility, EPISKIN implements a globally harmonized quality system across its production units in France, China, and Brazil. This system includes rigorous quality control of raw materials, standardized operating procedures, and comprehensive characterization of each RhT batch. Quality control, implemented at three distinct time points (during production, at shipment, and 7 days post-shipment), encompasses viability, histology, and barrier function assessments, each with appropriate positive and negative controls. These assessments ensure consistent tissue structure, barrier properties, and biological function. In 2024, EPISKIN produced over 100,000 units across its global sites, maintaining a > 98% conformity rate with OECD guidelines. Comprehensive user training and technical support further ensure the responsible use of EPISKIN RhT models. This globally standardized production process enhances RhT model reliability and promotes wider adoption of these *in vitro* testing strategies, ultimately leading to more reliable and ethically sourced data for safety and efficacy assessments.

Presentation: Oral

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Expanding access to NAMs with free toxicology training

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There is a growing understanding that New Approach Methodologies (NAMs) provide many benefits over traditional animal-based methods, yet most toxicological curriculum still focuses on outdated animal-based methods. In order to replace animal-based methods, regulators and legislators need clear demonstrations that these methods can outperform the animal-based methods they were trained with before they can have the confidence to accept NAMs. Meanwhile, industry needs to see the regulators accepting NAMs before they prioritize and implement them in their regulatory applications.

The lack NAMs implementation leads to an important educational gap in the field of toxicology, as new toxicologists are still being trained in outdated methods and rely on self-education to modernize their approaches. To bridge this dilemma, the NAM Use for Regulatory Application (NURA) program provides free training on human-relevant methods that can replace the traditional animal-based methods.

Additionally, global access to affordable training remains a barrier. NURA aims make training more accessible by providing free training to companies, scientific societies, regulatory bodies, or anyone else who needs it. NURA offers in-person trainings such as seminars, hands-on trainings, or CE courses, as well as virtual trainings that are made available on-demand for anyone to access despite scheduling or time zone conflicts. NURA's on-demand courses now include Spanish and English subtitles to increase accessibility.

NURA bridges the educational gap between the outdated, animal-based methods taught in current toxicology curriculum and the availability of more modern, human-based approaches by providing accessible hands-on training to anyone who wants to replace animals in their chemical assessment.

Presentation: Oral



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The big picture and conceptualization of the problem formulation

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A number of global initiatives are exploring how best to incorporate exposure-driven risk assessments into the regulatory frameworks for (agro)chemical safety assessments, incorporating new approach methods into a more human relevant and efficient process. How this looks in practice is often questioned, and all stakeholders need to build their experience and confidence in the new methods to appreciate the benefits of the 3Rs. A key feature to ensure a robust and fit-for-purpose risk assessment is to understand which types of information or data are needed to address the problem formulation. This session begins by presenting how relevant information can be leveraged to minimize the need to generate further animal data for a safety assessment.

Presentation: Oral

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GIVIMP and its role in *in vitro* regulatory testing

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The OECD guidance on “Good *In vitro* Method Practices” GIVIMP serves as a framework for developing and implementing *in vitro* methods, thereby enhancing the credibility and regulatory acceptance of New Approach Methodologies (NAMs). The OECD Principles of GLP function as a comprehensive managerial quality control system for non-clinical health and environmental studies. Studies intended for regulatory submission must follow GLPs; however, GIVIMP may be used as important supplemental guidance by providing nuanced information for *in vitro* method users that extends beyond the GLPs and the OECD advisory document on the Application of the Principles of GLP to *In vitro* Studies. This presentation will provide insight into why laboratories, including those that are GLP compliant, will benefit from assessing their procedures against the recommendations in GIVIMP. Specific examples will be used to show where GIVIMP recommendations can help meet the requirements for GLP studies. The presentation will also discuss the important role that GIVIMP plays in guiding the development of methods that are intended for answering regulatory questions. It will show how following GIVIMP aligns your work with the framework for establishing scientific confidence in new approach methodologies as published in Archives of Toxicology (2022, van der Zalm, et al). When laboratories follow the GIVIMP recommendations they are aligned with regulatory expectations for NAMs and are supporting improved regulatory acceptance of *in vitro* methods.

Presentation: Oral



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Patient-derived organoids for precision medicine in breast cancer: A study in Brazilian patients

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Patient-derived organoids (PDOs) are advanced 3D *in vitro* models that replicate tumor architecture, biological composition, and microenvironment complexity, making them valuable tools for cancer research. In our breast cancer organoid study, 60% of the 125 breast cancer tissue samples processed as PDOs were successfully cultivated, demonstrating the feasibility of using these models for further investigation.

We selected four cases representing the major breast cancer subtypes – Luminal A (LUM A), Luminal B (LUM B), HER2+, and triple-negative (TNBC) – and screened them against 118 compounds and 16 therapeutic regimens currently used in Brazil's Unified Health System (SUS). Organoids were cultured until passage 4, manually plated in 384-well plates, and treated with compounds prepared using an acoustic dispensing system (ECHO) and applied via automated liquid handling (Janus MDT, Perkin Elmer, HTS platform). Organoid viability was assessed by luminescence (CellTiter-Glo3D, Promega) after six days. Drug responses were evaluated using growth rate (GR) inhibition calculations to determine GR50 values (drug concentration causing 50% growth inhibition).

Several drug combinations demonstrated strong cytotoxic effects across all subtypes, including Doxorubicin (DXO) + Cyclophosphamide (CP) (GR: -0.54 to -0.97), DXO + CP + Docetaxel (DTX) + Trastuzumab (TrZ) (GR: -0.66 to -0.97), DXO + CP + Paclitaxel (PTX) + Carboplatin (GR: -0.65 to -1.00), and DXO + CP + PTX + TrZ (GR: -0.68 to -1.00). For TNBC, additional cytotoxic responses were observed with capecitabine, cisplatin + gemcitabine, and DTX + TrZ + pertuzumab (GR: -1.00 for all). GR50 values were validated (r^2 : 0.95-1.00, $P < 0.05$) for 10 compounds in TNBC, 4 in HER2+, 12 in LUM A, and 10 in LUM B. Notably, ABT-263 (Bcl-2 inhibitor), BMS-754807 (ILGF1 inhibitor), and Lapatinib (ErbB1 e ErbB2 inhibitors) showed broad efficacy across subtypes.

Our findings highlight subtype-specific drug responses, with Luminal A and B showing strong responsiveness to hormonal therapy, while TNBC exhibited sensitivity to multiple cytotoxic regimens. Given TNBC's aggressiveness and the tendency for *in vitro* studies to overestimate drug efficacy, our study provides a framework for guiding personalized medicine by eliminating ineffective treatments and prioritizing the most promising therapeutic options. This research marks an important initial step toward precision medicine for breast cancer patients in Brazil, with the potential to generate clinically relevant insights and enhance therapeutic decision-making in real-world healthcare settings.

Presentation: Oral

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The experience of Anvisa using non-animal approaches in the toxicological assessment of pesticides

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The Brazilian Health Regulatory Agency (Anvisa) will present its recently published guidance on the toxicological evaluation of pesticides. The guidance recommends the use of new approach methodologies (NAMs) in a weight of evidence (WoE) framework. It will also provide examples from the pesticide reevaluation process conducted by the Office of Reevaluation of Pesticides within Anvisa's General Office of Toxicology.

For new technical pesticide products, Anvisa requires a list of traditional toxicological studies to assess the potential risks to human health (Resolution RDC 294/2019). However, toxicological studies may be waived if there is a valid technical justification. On the other hand, for pesticide reevaluation, there are no specific requirements, and all available studies are considered to reach a conclusion.

The latest versions of the guidelines of toxicological evaluation of pesticides, published on 23 December 2024 (Guides n. 66, 67, 68, and 69/2023 v.2), especially the guides on the potential of reproductive toxicity and of neurotoxicity of pesticides, already foresee the use of alternative approaches to animal testing. For endocrine disruption, the use of NAMs is already established, and Anvisa has guidance under preparation, which will be published soon.

In recognition of the challenges of traditional toxicity testing methods, a paradigm shift is underway in toxicology, from decisions based largely on *in vivo* animal testing using apical endpoints, to the increased use of *in vitro* tests (omics, cell-based, tissue-based) within the framework of Integrated Approaches to Testing and Assessment (IATA).

Especially for reproductive and developmental toxicity, the shift from complex *in vivo* tests to *in vitro* tests is more challenging. Due to its complexity, the entire process cannot be modeled in a single *in vitro* system; therefore, the different components of the reproductive cycle must be studied individually and then integrated into testing strategies.

Although *in vitro* methods cannot yet be used exclusively in a reproductive toxicity testing strategy for pesticides, the combination of tests in a stepwise and/or battery approach and their assignment to an Adverse Outcome Pathway (AOP) can provide relevant information on the hazard and risk of a substance.

The harmonization of procedures and the development of standard protocols with data interpretation are necessary to increase regulatory confidence and the implementation of alternative methods for toxicity assessment. Thus, at the current stage of method development and validation, when used in a WoE approach, this category of data can complement other lines of evidence (in animals and humans) and support regulatory decisions.

In the reevaluation carbendazim performed by Anvisa and published in 2022, there are in depth discussions in the context of AOPs that helped to reach the conclusions on the health hazards of this pesticide active ingredient (https://www.gov.br/anvisa/pt-br/acessoainformacao/pergunhasfrequentadas/agrotoxicos/reavaliacao-de-agrotoxicos-2/copy_of_NotaTcnicaFinaldeReavaliadoCarbendazimcomseusAnexos.pdf).

Presentation: Oral



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Advancing regulatory science: insights from the EPAA's NAMs User Forum 2024

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The European Partnership for Alternative Approaches to Animal Testing (EPAA) convened the "New Approach Methodologies (NAMs) User Forum" at the European Chemicals Agency (ECHA) in Helsinki, Finland, on October 30-31, 2024. This hybrid event brought together approximately 50 participants, including representatives from regulatory agencies, industry, non-governmental organizations (NGOs), academia, and European Union (EU) competent authorities. The forum aimed to delve into the application of NAMs in chemical safety assessment, with a particular emphasis on decision-making processes within tiered testing strategies.

Building upon the inaugural User Forum held in December 2023, this session sought to: share experiences and lessons learned from the regulatory application of NAMs, discuss advancements in tiered testing strategies integrating NAMs, and identify areas requiring further development to enhance the regulatory acceptance of NAMs.

The forum featured a series of presentations and case studies:

Regulatory perspectives: Insights were provided by the European Food Safety Authority (EFSA) and ECHA on the integration of NAMs into regulatory frameworks, highlighting both progress and existing challenges.

Developmental Neurotoxicity *In vitro* Battery (DNT IVB): Updates on the DNT IVB underscored its potential in assessing neurotoxicity without animal testing.

ASPIS-Initiated Safety Profiling Algorithm (ASPA): The ASPA initiative was presented as a promising approach for safety profiling, leveraging NAMs to predict chemical hazards.

Case Studies: Five case studies demonstrated the practical application of tiered testing strategies, combining various NAMs to strengthen evidence in chemical safety assessments.

A focal point of the discussions was the evolution of tiered testing strategies, structured into three tiers encompassing hazard identification, exposure assessment, and toxicokinetic evaluation. The forum addressed the determination of the appropriate types of NAMs (*in silico* and *in vitro*) applicable at each tier and the establishment of criteria to assess the reliability of NAM-derived data in regulatory decisions.

The NAMs User Forum highlighted significant progress in the development and application of NAMs within tiered testing strategies. However, it also identified the need for continued efforts to enhance the acceptance and integration of NAMs in regulatory contexts, develop robust frameworks for decision-making based on NAM data, and to foster collaboration among stakeholders to address existing challenges and knowledge gaps. These insights are pivotal for advancing the adoption of NAMs, aligning with the EPAA's commitment to promoting alternative approaches to animal testing in regulatory science.

Presentation: Oral

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A framework for assessment of certainty on *in vitro* to *in vivo* extrapolation for environmental risk assessment

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Environmental risk assessment is a procedure to achieve the protection of biodiversity with all species. In contrast, toxicity data are only available for a few standard species that can be tested in the laboratory and for which global testing guidelines from the OECD exist. Currently, the main assumption when conducting an environmental risk assessment is that this standard species will protect local biodiversity globally, which is addressed by safety factors. Therefore, the aim of replacing this standard species with alternative methods in the current risk assessment scheme is too short-sighted. We should not be asking how good a method needs to be to replace, for example, a study on bird reproduction, but rather we need to address how well this alternative method asks the risk assessment questions for the protection of all bird species. Differences in species sensitivity can occur due to intrinsic sensitivity (sensitivity at the target site), differences in toxicokinetics (uptake, distribution, and elimination), and exposure due to different behaviors (e.g., feeding patterns). Mechanistic modeling has high potential for extrapolating effects from *in vitro* to *in vivo* and across species. However, currently, different approaches with varying degrees of complexity are used for the same purpose, and very often only successful examples are presented. In this talk, we will present a framework to classify approaches for *in vitro* to *in vivo* extrapolation, taking species extrapolation into account, and making implicit assumptions explicit. The proposed framework allows for comparing different approaches with regard to the mechanisms included. We will present examples of successful mechanistic extrapolation for fish, amphibians, and birds and compare the safety of these mechanistic approaches based on non-animal methods with the uncertainty of the traditional *in vivo* approach to biodiversity risk assessment. For fish, the examples use a combination of cell testing and physiologically based toxicokinetic-toxicodynamic modeling to predict adverse effects on growth as well as key events such as vitellogenin induction. For amphibians, the critical body burden approach was used to extrapolate from *in vivo* fish data to overspray events of terrestrial amphibian live stages, thus protecting amphibians without the need for amphibian testing. For birds, the example uses an embryo test in combination with physiologically based toxicokinetic modeling to predict residue concentrations in the egg and dynamic energy budget modeling to simulate the effects on the embryo. All these approaches complement the question of risk assessment itself, rather than simply replacing a particular assay. The presented framework will enable a better assessment of the safety of an approach to answer a given risk assessment question and highlight the advantage of alternative methods over *in vivo* approaches.

Presentation: Oral



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An integral approach to replace animal-derived products

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Advanced *in vitro* methods are progressively being recognized as promising alternatives to animal studies. However, these models are often not completely animal-free. Many of them rely on the use of animal-derived products (ADP) such as fetal calf serum (FCS) and basement membrane extracts (BME; like Matrigel). Moreover, animal-produced antibodies are widely used for downstream analyses. The use of ADP is associated with ethical and scientific concerns, highlighting the need for more reproducible, animal-free alternatives. Therefore, one of the focus points of the 3Rs Centre Utrecht (3RCU) is to replace/reduce the use of ADP for biomedical cell culture purposes.

To do so, the 3RCU hosts and manages the publicly available 3Rs Research Tools program, which includes the FCS-free Database (<https://fcs-free.sites.uu.nl/>) and the recently developed BME-free Database (<https://bme-free.sites.uu.nl/>). As we will demonstrate, these databases provide a comprehensive overview of alternative products and contain a search tool with multiple filter options allowing researchers to finetune their search to their specific needs.

In addition, we organize a working group dedicated to the local replacement of ADP at the Utrecht Science Park. To facilitate this, we coordinate central storage and free supply of alternative FCS products. We have also collected data on the annual ADP use at the Utrecht Science Park over the last 10 years, providing eye-opening data on invisible animal use and associated suffering. In addition, we will assess the environmental impact of the production of ADP and compare these to non-animal alternatives.

To reduce the use of animal-produced antibodies, we are collaborating with companies that produce recombinant antibodies enabling researchers access to free test samples. Finally, we actively use LinkedIn for targeted campaigns to create attention, awareness, and adherence.

We hope that this integral approach ultimately leads to a reduction in the use of ADP (and associated invisible animal use) and thereby facilitate the transition to truly experimental animal-free science.

Presentation: Oral

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The 3Rs Centre Utrecht: Bridging NAMs and the 3Rs framework

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The mission of the 3Rs Centre Utrecht (3RCU) is to stimulate the development, acceptance and implementation of methods that can Replace, Reduce or Refine animal experiments. To do so, we try to form a bridge between researchers working with New Approach Methodologies (NAMs), and researchers that are using experimental animals.

The 3RCU is embedded within the Animal Welfare Body Utrecht, which supervises the welfare of laboratory animals and the quality of animal experiments in research and education, providing us with direct access to researchers that are using, or that are planning to use, experimental animals. We will demonstrate how we reformulated the questions relating to the use of the 3Rs principles in project proposal forms to reduce repetitive and reactive responses. Close collaborations with the EU COST Action IMPROVE, as well as with the TOGETH3R network, extend our reach and provide us with an international scope.

We aim to maximally facilitate researchers when they are considering *in vitro* methodology instead of, or complimentary to, *in vivo* approaches. We host free digital tools such as the fetal calf serum- and basement membrane-free cell culture database, are setting up a helpdesk to facilitate access to NAMs expertise and communicate on recent 3Rs developments via social media as LinkedIn. Our strategy encompasses stimulating parallel *in vitro-in vivo* studies, using targeted campaigns via social media to create adherence, and reformulating the 3Rs principle in project applications. Through the organization of working groups, internships, centralization of resources and bench space we aim to generate data in addition to ideas. We will showcase the setup as well as the results of this integral approach, which makes the 3RCU stand out as an interesting partner for both researchers working with NAMs as well as for researchers working with experimental animals.

Presentation: Oral



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Filling the gaps between basic, translational, and clinical research: a systematic review and meta-analysis in animal models of *Trypanosoma cruzi* infection

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Systematic reviews (SR) and meta-analyses (MA) play a crucial role in advancing the 3Rs in animal research, allowing for the formal synthesis of existing data and assessing the global effect of interventions without the need for new animal experiments. SR and MA contribute to more ethical animal research by enhancing the methodological quality of animal studies, supporting evidence-based decisions and facilitating the translation of preclinical findings to clinical trials.

Chagas disease remains a significant global health concern, yet treatment options are limited to benznidazole (BZ) and nifurtimox (NFX), which are far from ideal. The lack of standardized and refined animal models may contribute to the limited success of drug development. Several sterol 14-demethylase inhibitors (CYP51), including azoles (i.e., posaconazol (POS), ravuconazol (RAV) and itraconazol) showed promising results in *in vitro* and *in vivo* models, but failed in clinical trials. We hypothesize that the failure of these therapies in clinical settings may be attributed, in part, to the inadequate interpretation of preclinical studies, particularly in terms of their scope and limitations.

In this context, a series of SR and MA were conducted to evaluate the effect of CYP51 in animal models infected with *T. cruzi*. Data including animal models, parasite strains, treatment regimens, and cure rates was extracted and summarized and primary outcomes, such as maximum parasitemia values, survival, and parasitological cure, were included in the meta-analysis when available.

Animal models exhibited a wide range of variability, regarding mice and parasite strains, treatment onset, and posologies, among other characteristics. Also, most studies exhibited an uncertain risk of bias. Animals treated with itraconazole, RAV, or POS survived significantly longer than untreated infected animals, with no significant difference compared to animals treated with positive control drugs (RR = 1.01 [0.98, 1.04], P = 0.54). Additionally, the overall analysis indicated that

RAV or POS was less likely to achieve parasitological cure compared to BZ or NFX (Odds Ratio [OR] = 0.49 [0.31, 0.77], P = 0.002), confirming the parasitostatic effect observed in later clinical trials.

This SR and the MAs shed light on the reasons behind the failure of azoles in clinical trials and underscores the need to improve animal models of *T. cruzi* infection. Refining these models and filling the gaps between basic, translational, and clinical research, facilitates the development of more effective therapies for Chagas disease.

Presentation: Oral

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Phasing out *in vivo* testing for veterinary vaccines: A global endeavor with key local and regional strategies

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Regulations require veterinary batch-release testing to confirm vaccine potency and safety, but some of those tests have traditionally relied on large numbers of laboratory (and non) animals. Advances in vaccine research and development, products characterization and state of the art quality control strategies offer increasing opportunities to replace *in vivo* testing for the release of veterinary vaccines. Significant progress in transforming the quality control release strategies allowed manufacturers to reduce reliance on animal testing, and some regulatory authorities to remove or substitute animal testing from their requirements [1]. The presentation provide an overview of the available opportunities that have transformed the release strategies of some veterinary vaccines, and the regulatory acceptance of non-animal testing opportunities and will present the ongoing efforts from the Animal Free Safety Assessment Collaboration and HealthforAnimals in their joint project to include future proof language in the World Organization of Animal Health Terrestrial Manual and support local regulatory transformation (Link to the last webinar organized by the two organizations: https://www.afsacolzlaboration.org/sciencex_event/webinar-transition-to-non-animal-based-veterinary-vaccine-batch-release-testing-policy-and-regulations-theoretical-aspects-and-case-studies/).

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Presentation: Oral



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Implementation plans for replacement of animal testing for human vaccines

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Today, strict quality control standards for vaccine production and release testing enable use of innovative approaches that can fully substitute animal testing and ensure batch-to-batch consistency, as well as ever higher quality, safety and efficacy. Significant steps have been done by manufacturers in both High-Income Countries and Low- and Middle-Income Countries in transitioning away from traditional animal-based testing, and the same effort has been made by some regulatory authorities and their national control laboratories. The presentation provide an overview of the Humane World for Animal project to support manufacturers, regulatory authorities and national control strategies to plan their transformation and possible investment on non-animal testing approaches, and understand how to overcome economic, technical, regulatory and other barriers to the removal, waiving and substitute obsolete animal testing. The project (link: <https://www.afsacollaboration.org/new-assays-implementation-and-acceptance/>) created knowledge sharing and discussion opportunities with key experts on the developed and in development *in vitro* approaches (link: <https://www.afsacollaboration.org/event/>), supported its participants to prioritize and plan the implementation of *in vitro* approaches, and facilitate the alignment of agendas of many international organizations.

Presentation: Oral

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Advancing medical device safety assessment with NAMs in a complex regulatory landscape

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Following our workshop on NAMs for medical devices (Kerecman et al., 2017), we review the significant advances in this field against the backdrop of a rapidly evolving market and regulatory landscape. The global medical device market has expanded to approximately US\$650-700 billion, with an annual growth rate of 5-6%. In 2023, the FDA authorized a record 124 new medical devices, highlighting the increasing complexity of the safety assessment challenge. Modern medical devices can contain thousands of chemicals – a recent UNEP report identified over 13,000 chemicals associated with plastics alone, with more than 3,200 having hazardous properties.

This complexity underscores the urgent need for comprehensive safety testing approaches beyond traditional animal models. Micro-physiological Systems (MPS), including organ-on-a-chip technologies, represent a transformative approach that provides enhanced human relevance, dynamic testing conditions, and integrated tissue responses. These systems can more accurately model the complex interactions between devices and human tissues, capturing both immediate and long-term effects of device materials, leachables, and wear particles.

Artificial intelligence further enhances these alternative testing paradigms by enabling *in silico* trials that simulate device performance under thousands of conditions, predictive modeling for early identification of potential toxicity profiles of chemicals and increasingly materials, automated analysis of safety documentation, and real-time post-market surveillance to detect early safety signals. AI-driven digital twins that create virtual patient replicas offer particularly promising applications for personalized device safety assessment, while federated learning enables multi-center data sharing without compromising patient privacy.

However, significant challenges remain in applying these technologies to solid materials testing, including: accurately replicating surface-tissue interactions, modeling chronic exposure scenarios, simulating real-world mechanical forces, and standardizing protocols for regulatory acceptance. Additionally, the increasing sophistication of medical devices requires new safety assessment paradigms.

Given the dominance of the US market, we propose an FDA Modernization Act 3.0 framework that would extend the NAMs integration approach from pharmaceuticals to medical devices, establishing clearer regulatory pathways for alternative testing methods. This paradigm shift would not only address ethical concerns by reducing animal testing but also improve predictive accuracy, streamline regulatory processes, and ultimately enhance patient safety in an increasingly complex medical device ecosystem.

Presentation: Oral



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NAMazing: Next generation validation for next generation risk assessment

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Next generation risk assessment (NGRA) requires a fundamental transformation in toxicological test method validation. Traditional validation approaches, designed for animal tests and simple *in vitro* methods, are increasingly inadequate for evaluating complex New Approach Methodologies (NAMs), artificial intelligence-based approaches, and integrated testing strategies. This paper presents a comprehensive framework for “next generation validation” that leverages artificial intelligence and modern computational capabilities to create more efficient, thorough, and dynamic validation processes.

The proposed framework emphasizes human relevance over simple concordance with animal data and introduces key innovations including e-validation, mechanistic validation, and post-validation companion AI agents. E-validation employs sophisticated algorithms for reference chemical selection, study simulation, and continuous performance monitoring, while mechanistic validation evaluates whether methods accurately capture relevant biological pathways and mechanisms of toxicity. The new validation approaches go hand-in-hand with needs for reporting standards and data sharing practices.

The validation of AI-facilitated NAMs faces unique challenges, including addressing the “black box” nature of complex AI systems through explainable AI approaches, ensuring reproducibility across different computational environments, and maintaining regulatory acceptance while embracing innovation. Standards for documentation, versioning and applicability domains / context of use need to be developed. The paper addresses these critical implementation challenges along with data quality standardization and international harmonization, providing specific recommendations for various stakeholders.

Looking forward, validation will increasingly embrace dynamic, adaptive approaches that evolve alongside scientific understanding and technological capabilities. The integration of artificial intelligence will enhance analysis of complex data, enable real-time monitoring of method performance, and support more sophisticated uncertainty quantification. Success in this transformation requires coordinated effort across regulatory agencies, industry partners, and academic institutions. The proposed framework represents a crucial step toward more efficient and accurate chemical safety assessment while maintaining necessary standards for public health protection.

Presentation: Oral

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Advancing next-generation risk assessment for cosmetic ingredients: Transferring the 10-step read-across framework from human to environmental safety

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Next-Generation Risk Assessment (NGRA) aims to establish scientifically robust safety evaluations without reliance on animal testing. While NGRA frameworks have been successfully developed for human safety, their application in environmental safety remains largely unexplored. This study demonstrates the transferability of the 10-step NGRA read-across (RAX) framework – originally developed for cosmetic ingredient safety (Alexander-White, 2022) – to environmental safety assessment.

The framework includes Tier 0 (Step 1: identifying exposure/use scenarios, Step 2: molecular structure identification, Step 3: collating supporting data & defining data gaps, Step 4: analogue identification & similarity hypothesis), Tier 1 (Step 5: systemic bioavailability/ADME, Step 6: supporting similar Mode/Mechanism of Action hypothesis), Tier 2 (Step 7: performing targeted testing), and the Assessment phase (Step 8: Read-across RAX to derive the point of departure (POD), Step 9: margin of safety (MOS) evaluation, Step 10: level of confidence assessment).

The 10-step framework has been successfully applied for human safety, with published case studies demonstrating its effectiveness for key ingredients such as parabens (Ouedraogo, 2022), phenoxyethanol (Hewitt, 2022), benzyl salicylate (Ebmeyer, 2024), and genistein / daidzein (Najjar, 2024). This study marks the first effort to apply this framework to environmental safety, using triclosan as a case study to assess its feasibility.

Applying the framework to triclosan, we evaluated environmental hazards, demonstrating that sufficient data can be leveraged to enable a structured, NAMs-based (New Approach Methodologies) assessment. Triclosan proved to be a suitable model compound for testing the framework due to the extensive ecotoxicological and mechanistic data available.

Applying NAM-based ecotoxicity testing, we assessed endocrine-related mechanisms of action, metabolic disruption, and oxidative stress responses in fish, invertebrates, and algae. PBPK models were incorporated, and read-across analogues were identified, demonstrating the feasibility of integrating mode-of-action (MoA) hypotheses into environmental NGRA.

This research provides strong evidence for transferring the NGRA framework from human to environmental safety, advancing risk assessment strategies across disciplines.

Our findings encourage industry, academic, and regulatory stakeholders to engage in this area, ensuring that non-animal, mechanistic-driven approaches become standard practice in environmental risk assessment. This transition highlights the broader adoption potential of NGRA, positioning this framework as a guideline for future assessments in environmental safety.

Presentation: Oral



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Assessing risk of bias in toxicological studies in the era of artificial intelligence

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Risk of bias is a critical factor influencing the reliability and validity of toxicological studies, impacting evidence synthesis and decision-making in regulatory and public health contexts. Traditional approaches for assessing risk of bias are often subjective and time-consuming. Our article (Archives of Toxicology, 2025) reviews key types of biases – selection, performance, detection, attrition, and reporting biases – in toxicological research and discusses specialized tools designed to address them, including SYRCLE and OHAT frameworks.

Recent advancements in artificial intelligence (AI) offer promising solutions for automating and enhancing bias detection. AI integration can significantly improve assessment efficiency, consistency, and accuracy. However, AI models themselves are susceptible to algorithmic and data biases, necessitating robust validation and transparency in their development. We highlight strategies for leveraging AI to screen studies, detect anomalies, and support systematic reviews, while proposing standardized methodologies, training, and policy implementation to mitigate biases in AI-driven analyses.

The integration of AI with risk of bias assessment tools presents a unique opportunity to address the increasing complexity in toxicological research. AI-based approaches enable more efficient screening of expansive datasets, detection of novel bias patterns, and development of predictive models that estimate bias likelihood based on study characteristics.

By adopting these advanced methodologies, toxicologists and regulators can enhance the quality and reliability of toxicological evidence, promoting evidence-based practices and ensuring more informed regulatory decisions, thereby advancing both human health protection and the reduction of animal testing.

Presentation: Oral

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AI in toxicology – The now, the new and the next

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The synergy of data generation and improvement of computers and algorithms has increased the power of AI more than billion-fold since AI was coined in 1956: Data in the world double every 18 month, i.e., 90% of all data were produced in last three years; computer double in capacity every 24 months (Moore's law) and AI algorithms double in capacity every 3 months since 2010. For most human skill tests, AI performs better than 90% of us and the latest LLM by Open AI achieved an IQ of 130+ in standard intelligence tests.

For toxicology, AI promises support for data retrieval, evidence integration (systematic reviews, risk assessments), predictive toxicology of untested compounds, digital pathology and support in reporting. The prospects of animal replacement with better accuracy in (human) prediction, ethical benefits and cost-effectiveness are enormous. Beyond this, accelerated assessments with automated data analyses, real-time monitoring and complex analyses come into reach with user-friendly prediction tools. These changes also promise to democratize knowledge, encourage open-access databases, algorithms and publications. As a copilot for toxicology, it empowers researchers, regulators, consumers and industry. The development toward such a co-pilote in the EU ON-TOX project is detailed here.

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Presentation: Oral



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AI in biomedical research and testing: Balancing innovation with ethical responsibility

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The integration of artificial intelligence (AI) into biomedical research and toxicology presents transformative opportunities while raising significant ethical concerns. This presentation critically examines the dual nature of AI applications, analyzing both their revolutionary potential and inherent risks. AI offers remarkable capabilities in predictive toxicology, mechanistic understanding, and regulatory applications, potentially accelerating the development of alternatives to animal testing. Successful implementations include automated read-across, probabilistic risk assessments, and high-throughput data analysis that enable more efficient chemical safety assessment.

However, these advances come with substantial challenges. Data biases can perpetuate or amplify existing inequities in biomedical research. The “black box” nature of complex AI models creates significant barriers to interpretation and validation. Additionally, regulatory frameworks struggle to adapt to rapidly evolving AI capabilities, creating uncertainty in implementation pathways. These challenges demand thoughtful approaches to ensure responsible AI adoption.

This presentation proposes a comprehensive framework for ethical AI implementation in biomedical research, emphasizing explainable AI (xAI) techniques that enhance transparency, robust validation frameworks that address potential biases, and ethical oversight mechanisms that maintain human accountability. By addressing both the opportunities and limitations of AI in biomedical contexts, we can leverage its capabilities while avoiding the pitfalls of technological overpromise (“AI snake oil”). The ultimate goal is to harness AI as a tool for advancing evidence-based toxicology and biomedical research while maintaining scientific integrity and ethical responsibility.

Presentation: Oral

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Evaluation of two alternative training methods in undergraduate laboratory animal science classes

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The use of animals in education remains common, though it has been refined significantly, particularly in alignment with the 3Rs principle, with a focus on humane and effective training methods. With this in mind, two alternative training models were developed to enhance the practical skills of undergraduate students in Veterinary Medicine from the School of Veterinary Medicine and Animal Science at the University of São Paulo, enrolled in the Laboratory Animal Science course, before performing procedures on rats and mice: a rat simulator and a silicon-based tail. The objective was to assess how these students perceived the use of the simulators. The evaluation focused on their well-being, practical techniques, and opinions regarding the realism and validity of these methods as learning tools. Additionally, the study aimed to determine the impact of using these simulators on students’ learning and performance, and whether they found them beneficial before working with live animals. To assess students’ perceptions, an original survey comprising 37 questions was developed, including a combination of demographic, qualitative Likert-scale, multiple-choice, and open-ended questions. A total of 54 responses were collected through the anonymous survey. The results were predominantly positive regarding the use of the simulators. Over 90% of students expressed light to strong agreement with the convenience of using both the rat simulator and the silicon tail, while more than 80% found them beneficial for learning the proposed procedures. However, opinions varied regarding the realism of the simulators. The rat simulator received lower approval for its skin resistance and overall appearance; approximately 60% of students agreed it was realistic, while about 20% strongly disagreed. In contrast, the silicon tail was better received, with over 90% of students finding its appearance realistic and approximately 70% agreeing that its resistance was accurate. The clarity and purpose of the instructions for using the models received unanimous agreement (100%), which may be attributed to the overall satisfaction with the training experience. One question in particular followed Bloom’s taxonomy to analyze students’ perception of the learning process. The results indicated that up to the third level – applying the techniques – students aligned with Bloom’s framework. However, when it came to the stages of analyzing and evaluating, they reported feeling more confident in their assessment skills than in applying the techniques they practiced. This suggests that students found performing procedures more challenging than observing and evaluating a third party’s actions. This outcome could be attributed to the heightened sense of responsibility they experienced when handling live animals. Overall, the models were considered realistic, although



some suggestions for improvement were made, particularly regarding the rat simulator. Additionally, students reported feeling less anxious when practicing with the models and more confident after the activities. While there is room for improvement in both the simulator and their application, they represent a promising refinement method. The simulators demonstrated positive results and received overall approval from students. The goal is to expand alternative training methods in universities, foster new ideas, and improve animal welfare and the effectiveness of education with live animals.

Presentation: Oral

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Next-generation full-thickness human skin models produced using 3D electrospun scaffolds and animal component-free culture media

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Background: *In vitro* full-thickness human skin models are important for testing cosmetics, chemicals and new pharmaceuticals. However, these models commonly utilize animal-derived collagen as a main element of the stromal matrix. Additionally, culture media used to produce these models commonly contain undesirable animal-derived components including fetal bovine serum (FBS) and bovine pituitary extract (BPE). To address these shortcomings, we developed full-thickness skin models without animal-derived collagen using electrospun scaffolds as structural components, together with open-source FBS/BPE-free culture media formulations.

Methods: (Bio-SpunTM) electrospun scaffolds are composed of randomly oriented fibers with diameters similar to that of native extra-

cellular matrix. Scaffolds were attached to Transwell[®] inserts in place of typical microporous membrane supports. To produce the dermal component, Bio-SpunTM scaffolds were seeded with primary human dermal fibroblasts and cultured under submerged conditions in FBS/BPE-free medium Primary human epidermal keratinocytes were then seeded onto the stromal components. The constructs were cultured at the air-liquid interface (ALI) in FBS/BPE-free ALI epidermal differentiation medium (open-source, BioSurfaces) to produce fully developed 3D full-thickness epidermal tissue models. Histochemical and immunohistochemical staining, and transmission electron microscopy (TEM) were utilized to evaluate morphological and ultrastructural features. Tissue barrier was evaluated by measuring Triton X-100 penetration using a time-to-toxicity (ET50) assay.

Results: H&E-stained paraffin sections revealed robust stromal components populated with viable fibroblasts. The fibroblasts proliferated within the synthetic scaffolds and synthesized native collagen and extracellular matrix materials that self-assembled to produce robust and stable stromal matrices within 3-6 days. Immunohistochemical staining revealed uniform collagen I deposition throughout the entire electrospun dermal component. TEM of the dermal-epidermal junction showed large numbers of striated collagen fibers and a well-developed basement membrane. H&E-stained paraffin sections also showed well-developed stratified epidermis by Day 10 following ALI culture, consisting of basal, spinous, granular and stratum corneum components. TEM of the stratum corneum showed abundant production and deposition of lamellar lipid sheets between the corneocytes. A viable epidermal layer with stable thickness was maintained for at least Day 35 after ALI (longest timepoint evaluated to date), providing an extended window of useful downstream experimentation time. The production protocol was successfully transferred to an independent research laboratory (IIVS), where tissues with comparable histology were readily produced. Functional barrier assessment conducted by IIVS determined an ET50 of ~13 hours, demonstrated a robust stratum corneum barrier.

Conclusions: Next-generation, *in vitro* full-thickness human skin models were produced using animal collagen-free 3D electrospun scaffolds and open-source FBS/BPE-free culture media formulations. The fully human skin models provide long-term stability and do not suffer from contraction and stromal degradation issues. These next-generation skin models offer promise for completely animal-product-free testing of cosmetics, chemicals, and pharmaceuticals.

Presentation: Oral



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EU roadmap to phase out animal testing for chemical safety assessments

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The European Union has a long-standing policy goal to phase out animal testing, if that is scientifically possible. With Commission Communication (2023)5041, the European Commission committed to prepare a roadmap to phase out animal testing for chemical safety assessments. Commission services started in July 2023 to develop the roadmap, with support of the European Chemical Agency (ECHA), the European Food Safety Authority (EFSA) and the European Medicines Agencies (EMA). The roadmap is intended to be adopted and published latest in the first quarter of 2026. It covers 15 areas of EU chemical legislation requiring currently animal testing for chemical safety assessments. The development of the roadmap goes along with a comprehensive consultation of stakeholders, including authorities, industry NGOs and the research community.

The presentation will provide details on the roadmap, which at the time of the 13th World Congress will be in its final drafting stage. The presentation will further indicate next steps for implementing the roadmap.

Already the Commission Communication announcing the roadmap contained an outline of its planned elements. The roadmap will provide guidance and recommend organizational changes and structures for moving towards chemical safety assessments in all relevant pieces of EU legislation on chemicals. It will contain an analysis and recommendations for implementing animal-free approaches in legislation, or for reduction or refinement of animal testing, where this is possibly in the short-term. It will also contain recommendations for the further developing of methods, including on extending their domain of applicability and necessary validation activities, as well as suggestions for combining methods to approaches. It will in addition outline a path towards completely phasing out animal testing in regulatory chemical assessments. For this long-term paradigm shift, it will be necessary to reconsider regulatory hazard identification, characterization and risk assessment approaches, including how to classify chemicals, with the aim to demonstrate the fulfilment of protection goals inherent to regulation with animal-free information only. The roadmap also intends to show up options to facilitate access to information on animal-free methods, or for reduction or refinement of animal testing, including guidance.

The roadmap further intends to provide recommendations for required organizational structures for efficiently moving towards phasing out animal testing for chemical safety assessments, which might involve the establishment of scientific working groups for animal-free approaches at agencies or the Commission. In addition, the roadmap will contain considerations on an improved decision system on method development taking into account regulatory needs and reflections on how to improve and accelerate standardization and validation, including its funding.

Finally, the Communication announcing the development of the roadmap emphasizes the international dimension for moving towards phasing out animal testing. Outreach to non-EU countries and multilateral organisations to foster the development and acceptance of non-animal testing methods is key for reaching the intended short- to long-term goals.

Presentation: Oral

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ToxCast bioassay-driven explainable AI for chemical hazard prediction: A bridge from research to regulation

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Advancements in New Approach Methodologies (NAMs) are transforming chemical hazard assessment by integrating mechanistic data into regulatory decision-making. This study presents an explainable artificial intelligence (AI) model that leverages ToxCast bioassay data to predict chemical hazards, demonstrating a practical pathway from research to regulation.

Our approach begins with a systematic analysis of ToxCast assays to assess their relevance to regulatory endpoints. By mapping bioassay data to apical outcomes, we identify key mechanistic insights that can inform regulatory toxicology. Building on this foundation, we develop AI models using advanced algorithms, including graph neural networks and multitask learning, to predict endpoints such as acute and developmental toxicity.

By integrating bioactivity profiles with adverse outcome pathways (AOPs), our model enhances interpretability and reduces the “black box” challenge, facilitating regulatory acceptance. Case studies illustrate how this AI-driven approach supports chemical prioritization and next-generation risk assessment (NGRA), providing a scalable framework for regulatory applications.

While ToxCast data offer a powerful resource for predictive toxicology, challenges remain in aligning *in vitro* bioassay results with *in vivo* outcomes. We discuss strategies such as quantitative bioactivity-concentration modeling and multi-dataset integration to address these limitations and improve regulatory utility.

This study exemplifies how AI-driven toxicology can transition from research to regulation, supporting the adoption of NAMs in chemical risk assessment. By leveraging bioassay data and mechanistic modeling, we provide a framework for more transparent, reliable, and efficient regulatory decision-making.

Presentation: Oral



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Facilitating science to policy transfer of NAMs for human and environmental assessment: An EU perspective

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The European Food Safety Authority (EFSA) is an agency of the European Union that provides independent scientific advice on matters related to food and feed safety, helping EU risk managers in making regulatory and policy decisions on food and feed safety.

EFSA's 2027 strategy for science, safe food and sustainability [1] emphasises the need to develop and integrate new approach methodologies (NAMs) and omics to advance its regulatory risk assessments. EFSA also contributes to the European Commission's Roadmap aiming at phasing out animal testing for chemical safety assessments [2]. NAMs offer several advantages in risk assessment, such as focusing on specific species of interest as well as providing insights on mechanistic understanding, kinetics and susceptible populations.

EFSA undertakes a number of projects to promote the regulatory use of NAMs and transition to next generation risk assessments. These projects include the use of artificial intelligence, computational modelling (e.g., PBK modelling, read-across), *in vitro* methods and integrated approaches of NAMs derived data for hazard and risk characterization. Furthermore, EFSA collaborates with various national and international stakeholders, including the WHO, OECD, sister agencies (ECHA, EMA, EEA) and other organisations, to address the challenges related to NAMs-derived data and their integration for feed, food and environmental risk assessment.

The presentation will outline some of EFSA's activities on NAMs and how these efforts inform decision-making with the view to protecting consumers, animals and the environment.

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Presentation: Oral

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Learning from the Global South

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In an era demanding rigorous scientific inquiry, the challenges of poor study quality and unreliable translatability persist, particularly impacting early career researchers. This talk, within the "Eye-opening" education for experimenters' session, explores how insights from the Global South, specifically through a refined ethical framework, can significantly enhance experimental design and ethical conduct.

The 12Rs Framework consists of domains: Animal Welfare (Replacement, Reduction, Refinement), Social Value (Respect, Responsibility, and Regulations), Scientific Integrity (Reproducibility, Relevance, and Transferability). The intersection of the three domains leads to Righteousness, Reliability, and Reckoning. The framework provides a holistic ethical compass for experimenters.

This talk will demonstrate how the teaching of experimental design for *in vivo* studies is led from an ethical lens in order to improve study quality and translatability of results to humans. This is emphasised through the Scientific Integrity domain of the 12Rs Framework. For example, the PREPARE guidelines are taught to ensure reproducibility through robust study design. The requirement for motivating the relevance of a study also compels experimenters to improve on their study design as they must consider and motivate the benefit of the study.

The talk will highlight that incorporating these perspectives can address the gap in understanding fundamental principles, leading to more robust, ethical, and translatable research. By adopting the 12Rs, researchers can ensure that their experiments are not only scientifically rigorous but also ethically sound and humane. This is particularly relevant to research in the Global South where access to technologically advanced *in vitro* alternatives is limited. The talk will be followed by an interactive session on how to apply the 12Rs Framework to the planning of animal research projects.

Presentation: Oral



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Advancing human-relevant *in vitro* models for developmental and adult neurotoxicity testing

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Developmental neurotoxicity (DNT) and adult neurotoxicity (ANT) testing is both currently performed in rats according to OECD (Organization for Economic Co-operation and Development) or US-EPA (Environmental Protection Agency) *in vivo* guideline studies. However, these approaches are highly resource-intensive and unsuitable for evaluating the vast number of chemicals requiring safety assessment, limiting their effectiveness in protecting human health. In response, regulatory bodies such as the European Food Safety Authority (EFSA) and the US EPA are actively supporting the development of *in vitro* methods, such as the DNT *in vitro* battery (DNT IVB), to enable faster, more efficient, and cost-effective neurotoxicity testing. As opposed to this, an ANT IVB systematically covering a panel of molecular initiating and key events relevant for ANT is currently missing.

As a key component of the DNT IVB, we have developed the Neurosphere Assay, which uses 3D cultures of human neural progenitor cells (hNPCs). This system models key neurodevelopmental processes, including hNPC proliferation, differentiation into neurons, astrocytes, and oligodendrocytes, cell migration, and neurite outgrowth.

Additionally, we established a human cell-based neural network formation (hNNF) assay that includes hiPSC-derived excitatory glutamatergic and inhibitory GABAergic neurons, along with primary human astrocytes, cultured on 48-well microelectrode arrays (MEAs).

For ANT assessment, we introduced the BrainSphere model, a 3D system that enables the study of acute and chronic neurotransmitter receptor effects using 96-well MEAs and spike sorting techniques.

Our findings demonstrate the successful implementation of the Neurosphere assay for multi-endpoint evaluation and support the use of these DNT IVB models for chemical screening and mode-of-action (MoA) analysis. These results can be integrated into Adverse Outcome Pathways (AOPs) to support regulatory decision-making.

For ANT, we validated the BrainSphere approach by targeting key neurotransmitter receptors – glutamate, GABA, dopamine, serotonin – and indirectly assessing acetylcholine signaling through acetylcholinesterase inhibition, forming a human multi-neurotransmitter receptor (hMNR) assay. A case study illustrates the assay's potential to identify specific ANT MoAs.

In summary, the presented DNT and ANT *in vitro* methods contribute significantly to advancing and standardizing alternative neurotoxicity testing strategies. By supporting the development of AOP networks, these assays pave the way toward broader regulatory acceptance of *in vitro* testing frameworks.

Presentation: Oral

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Key research initiatives in LATAM: Bridging human biomonitoring and computational toxicology

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Brazil stands as the world's leading coffee producer, where the extensive use of pesticides is economically critical yet poses health and environmental risks due to their non-selective mechanisms of action. Specifically, triazole fungicides are widely used in agriculture to manage fungal diseases and are known to disrupt mammalian CYP450 and liver microsomal enzymes. Computational toxicology refers to the use of computational tools to support toxicological research and chemical safety assessments via predictive modeling and complex data analyses for extrapolation and translation between streams of evidence, particularly human biology-based new approach methodologies (NAMs) that serve as alternatives to animal testing. This research establishes a framework for risk characterization of human exposure to triazole fungicides by internal-dose biomonitoring, biomarker measurements, and integration of high-throughput screening (HTS) data via computational toxicology workflows from the Integrated Chemical Environment (ICE). Three triazole fungicides, cyproconazole, epoxiconazole, and triadimenol, were detected in the urine samples of farmworkers and spouses occupationally and environmentally exposed to pesticides from rural areas (n = 140), from the southern region of Minas Gerais, Brazil, by gas chromatography coupled to mass spectrometry. Effect biomarkers analysis indicated significant alterations in the rural group (Kruskal-Wallis $p < 0.0001$), such as increased frequency of cellular changes associated with genotoxic effects by the oral mucosa cell cytome assay and oxidative imbalance, particularly among men occupationally exposed to pesticides. Additionally, elevated plasma bile acids indicated early and possibly reversible changes, as no significant differences were observed in liver enzyme levels (AST, ALT, and γ -GT). The hormones androstenedione and testosterone were significantly reduced in the farmworker group (Mann-Whitney $p < 0.0001$). In the ICE workflow, active *in vitro* HTS assays were identified for the three measured triazoles and three other active ingredients from the pesticide formulations. The curated HTS data confirm bioactivities predominantly related to steroid hormone metabolism, cellular stress processes, and CYP450 enzymes impacted by fungicide exposure at occupationally and environmentally relevant concentrations based on the *in vitro* to *in vivo* extrapolation (IVIVE) models. To characterize the risk associated with urinary triazole levels, Estimated Daily Intake (EDI) and Hazard Quotient (HQ) were calculated. The highest EDIs were observed for epoxiconazole, ranging from 0.47 to 6.31 $\mu\text{g}/\text{kg-bw}/\text{day}$ for men and 0.49 to 8.77 $\mu\text{g}/\text{kg-bw}/\text{day}$ for women in the exposed group. In the worst-case scenario, considering the highest detect-



ed urinary triazole value, the calculated HQ for epoxiconazole was 2.1 for men and 2.9 for women. These results characterize the potentially significant human health risk, particularly from the high frequency and intensity of exposure to epoxiconazole. This study showcases the critical role of biomonitoring and the utility of computational tools in evaluating pesticide exposure, as well as in continuing to build confidence in NAMs and translating toxicity information from HTS data to relevant human exposure scenarios in toxicological risk assessment studies.

Presentation: Oral

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The European Partnership on Alternative approaches to Animal testing (EPAA) Environmental Safety Assessment (ESA) project – Moving towards alternatives to animal testing for ESA

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Environmental Safety Assessments (ESA) are mandatory under multiple pieces of legislation in the EU and worldwide. Today, legislation is still relying largely on animal testing for ESA, despite alternative methods to animal testing becoming more and more available for several of the ecotoxicological endpoints required in legislation.

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a partnership between the European Commission and seven industry sectors that aims to promote the replacement of animal testing with innovative non-animal testing methods or approaches. In cases where alternative approaches are not available, EPAA advocates to reduce the number of animals needed or to refine animal testing (3Rs principle).

On 13/14 November 2023, EPAA organized the first Partner Forum meeting with more than 60 participants to perform a strategic, cross-sector review of the use of alternative approaches to animal testing for ESA in the European regulatory context. Based on the discussions, which indicated a huge potential for replacing animal testing in ESA, EPAA decided to establish a Project Team ESA with seven sub-groups covering different areas of ESA:

- 1) Fish acute toxicity
- 2) Bioaccumulation
- 3) Chronic aquatic toxicity
- 4) Endocrine Disruption
- 5) Mammals and birds
- 6) Validation of methods
- 7) Long-term paradigm shift to animal-free approaches

In total more than 40 experts in these seven subgroups gathered information on the current ESA requirements in all relevant pieces of EU legislation on chemicals in 2024 and 2025. The Project TEAM ESA systematically identified alternative approaches that are ready to be used for the ecotoxicological areas covered by the subgroups, that are in development or require validation. The team made further proposals for approach developments and identified research needs for cases where no alternatives are available. In particular for addressing chronic fish toxicity, endocrine disruption, or toxicity to mammals and birds, new approaches are needed. For completely phasing out animal testing in regulatory ESA, replacement of currently available methods addressing certain endpoints and species was seen as not feasible. Instead, regulatory environmental hazard and risk assessment requires a paradigm shift that re-considers the system of hazard identification, characterization on risk assessment, with the aim to demonstrate the fulfilment of protection goals inherent to regulation with animal-free information only. The Project Team identified steps and development needs for such a switch to a new regulatory system based on a Next Generation Risk Assessment scheme, including considerations on a generic bioactivity battery covering the most relevant environmental pathways, on methods for extrapolating from the sub-organism level to the populations or higher biological levels and the exploitation of advanced computational tools.

The work of the Project Team ESA resulted in seven conceptual papers containing the proposal of the subgroups. The presentation aims to provide an overview of the proposals and to indicate the next steps that will be taken to further progress and implement them.

Presentation: Oral



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Endocrine-disrupting potential of plastic additive chemicals on endangered species revealed by molecular docking analysis within the cross-species adverse outcome pathway framework

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Assessing plastic additive toxicity in endangered sea turtles is challenging due to ethical constraints on animal testing. This study demonstrates how molecular docking and AlphaFold protein structure prediction serve as effective alternative testing methods for cross-species toxicity extrapolation from humans to Korean loggerhead and green sea turtles.

Our alternative testing approach involved four steps: (1) protein sequence analysis using SeqAPASS to quantify conservation of androgen (AR) and estrogen receptors (ER) between humans and turtles; (2) generation of three-dimensional turtle receptor models using AlphaFold 3; (3) validation of cross-species extrapolation using ToxCast compounds; and (4) molecular docking simulations with AutoDock Vina to compare binding affinities of 150 plastic additives.

Results revealed high sequence conservation in ligand-binding domains, with binding affinity correlation coefficients reaching 97% between turtles and humans. This strong correlation indicates that plastic additives likely initiate toxicity through identical molecular mechanisms across species. While most compounds showed similar binding patterns, estradiol and Tris(2-chloroethyl) phosphate (TCEP) exhibited higher binding affinities to turtle receptors than human receptors, suggesting potentially greater toxicity risks to these ecological species.

This computational approach demonstrates how alternative testing methods can effectively predict chemical susceptibility in protected species without direct testing. By identifying both shared toxicological pathways and species-specific vulnerabilities, this methodology supports the 3Rs principles (Replacement, Reduction, Refinement) while generating valuable data for evidence-based conservation and human health assessment. Our findings highlight the need for species-specific risk assessment strategies for compounds that may disproportionately impact wildlife compared to humans.

This work was supported by Korea Environment Industry & Technology Institute (KEITI) through Technology Development Project for Safety Management of Household Chemical Products, funded by Korea Ministry of Environment (MOE)(RS-2023-00215309).

Presentation: Oral

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Introduction to the global education hub for animal-free innovation: Background and progress

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As Nelson Mandela eloquently said: “Education is the most powerful weapon which you can use to change the world.” Education is a key but underestimated element in the transition to animal-free innovation. This applies to stakeholders of all generations and career levels in the broad field of life sciences, including biomedical research, veterinary science, pharmacology, and toxicology.

An extensive array of non-animal methods is evolving, from advancements in cell culturing, such as organ-on-a-chip and organoid technologies, to *in silico* applications, including sophisticated computer simulations and data mining. These novel methods will only be developed and used more broadly if students and professionals learn – on a large scale – how to develop, create, apply, and evaluate them.

To promote the global transition to humane teaching and training and to teaching non-animal methods in biomedical research, the Global Education Hub for animal-free innovation was initiated in 2023 by the Transition Programme for Animal-free Innovation Utrecht and PETA UK. Through a series of interactive hybrid meetings, we set out the vision, mission, values, and goals of the hub with representatives from academia, industry, government and NGOs representing 17 countries. A meeting report about the hub is being prepared for publication.

The hub’s mission is to accelerate the transition to animal-free innovation by putting education, training and communication at the center of all hub-related activities. It aims to connect and inspire individuals, organizations, and companies by co-creating, sharing, and disseminating knowledge, educational tools, resources, and materials about non-animal methods for life sciences research, testing, teaching, and training. This hub aims not only to foster scientific excellence, collaboration, and global impact in using non-animal methods but also to address the ethical concerns and limitations of using animals for research, testing, teaching and training.

Three working groups have been established within the hub, each with a different target audience for education: high school and Bachelor/Master, PhD and Postdocs, and regulators.

After an introductory presentation on the background of the Global Education Hub and the progress of the three working groups, we will have an interactive discussion with the audience for the work of the hub by using an open mic and tools such as Slido and Word Cloud. The discussion will last an hour and will be moderated by the session chairs.

We hope to attract participants from academia, industry, government, and non-governmental organizations to this workshop to discuss what is necessary to establish human-based, animal-free science as a strategic topic in education. We also aim to further grow the Global Education Hub, in terms of educational and geographical coverage.

Presentation: Oral



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Pioneering animal-free careers: Ethical pathways in biomedical research

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The landscape of biomedical research is undergoing a profound transformation as the scientific limitations and ethical concerns of animal experimentation become increasingly recognized. As human-relevant methodologies emerge, new career opportunities are opening for students and professionals in biology, veterinary medicine, and human medicine who seek to align their work with ethical values and scientific innovation. This talk explores career pathways that prioritize cutting-edge, animal-free approaches and offers practical strategies for navigating this evolving field. A key aspect of this transition is the advancement of innovative, non-animal methodologies that enhance scientific rigor and human relevance. Breakthroughs such as microphysiological systems, 3D bioprinting, and AI-driven modeling are revolutionizing drug discovery, disease research, and toxicity testing. These developments not only address ethical concerns but also offer promising career avenues for those committed to humane and effective biomedical research. The presentation will provide an overview of career opportunities across multiple sectors. In academia and research, professionals can advance non-animal methodologies through teaching and experimental work, shaping the next generation of scientists. Regulatory agencies need experts to develop and implement policies that integrate animal-free models in biomedical and toxicological assessments. The pharmaceutical and biotechnology industries increasingly adopt human-relevant approaches in drug development and personalized medicine, while medical and veterinary professionals lead the shift toward simulation-based training and other humane teaching methods. Advocacy organizations and nonprofits play a crucial role in raising public awareness and influencing policy to support the widespread adoption of alternatives. For those seeking careers in animal-free science, several key strategies can facilitate this shift. Identifying relevant education and training programs is essential for building expertise. Engaging in professional networks within academia, industry, and regulatory spaces provides valuable connections and opportunities. Developing advocacy skills helps individuals navigate resistance within traditional biomedical fields and drive systemic change. Despite the growing demand for animal-free innovations, institutional inertia and regulatory barriers remain significant challenges. This talk will offer insights into policy developments, interdisciplinary collaboration, and steps to advance humane alternatives. Understanding these challenges and opportunities will better equip participants to pursue impactful careers. Ultimately, this session aims to empower early-career scientists and professionals to align their careers with ethical integrity and scientific excellence. By embracing and promoting humane methodologies, they can contribute to a more ethical, effective, and sustainable biomedical future.

Presentation: Oral

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Replacing animals for rabies diagnosis in Brazil

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Rabies is a lethal viral disease with a nearly 100% fatality rate once symptoms appear, making rapid and accurate diagnosis crucial for outbreak control and prevention. This work provides an overview of advancements in animal replacement for rabies diagnosis, focusing on alternative methods, transition experiences in Brazil, regulatory challenges, and the global shift away from live animal use. When a sample from a potentially rabies-infected animal tests negative, a confirmatory test is required. The traditional method, the Mouse Inoculation Test (MIT), involves injecting brain tissue into live mice and monitoring them for weeks. Despite being costly, slow, and causing significant animal suffering, MIT remains Brazil's confirmatory standard. Among 41 official Brazilian laboratories conducting rabies diagnosis, only three have successfully replaced animal-based tests. The others have not publicly disclosed transition plans or results. This is concerning given that alternative methods, accepted by the WOAHP, offer faster, humane, and highly sensitive results. These include conventional and quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR, RT-qPCR) and the Rabies Tissue Culture Infection Test (RTCIT), internationally validated as MIT replacements. A key example is the Central Laboratory of Paraná (Lacen-PR), the first in Brazil to fully transition to RT-qPCR for rabies diagnosis. This shift reduced costs, cut processing time from 26 to 4 days, and increased testing capacity, demonstrating efficiency, ethical benefits, and public health advantages. Cost analyses confirm that alternative methods are more economical than MIT, suggesting its persistence in Brazil is due to regulatory uncertainties, limited transition resources, and adherence to traditional methods. These barriers have hindered progress, but the upcoming 2025 regulatory change led by the National Council for the Control of Animal Experimentation (CONCEA) toward recognizing four alternative rabies diagnosis methods is expected to drive a major shift. Beyond ethical and methodological advantages, alternatives offer a practical, logistically efficient approach, considering that few Brazilian laboratories meet strict animal-based confirmatory testing standards. Compliance requires extensive welfare protocols, strict biosafety, anesthesia and analgesia management, humane endpoints, and proper disposal, making it costly and labor-intensive. Many struggle to comply, leading some to abandon confirmatory testing, increasing false-negative risks in cases with low viral loads or degraded samples. Recognizing and adopting alternatives ensures confirmatory testing continues while improving efficiency, reducing costs, and aligning with technological and ethical advancements. Although transitioning to validated non-animal methods faces barriers and demands rigorous commitment, rabies diagnosis serves as a framework for replacing animal use in other disease diagnostics.

Presentation: Oral



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Questioning proposals with severe harm/high degree of invasiveness: Initiating processes toward banning

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This presentation explores strategies for questioning research proposals that involve severe harm or high degrees of invasiveness, emphasizing the necessity of ethical review mechanisms that recognize non-negotiable ethical limits. While ethics committees play a crucial role in evaluating protocols, there are still studies that validate extreme suffering without adequate justification, which raises concerns about the effectiveness of current harm-benefit assessments. We propose that review committees adopt a more structured approach, integrating tools like the MAPEPA (Method for Evaluating Proposals for Teaching or Research Involving Animals) to ensure rigorous ethical analysis. MAPEPA establishes a systematic scoring system that assesses whether a study aligns with ethical principles, including the principle of irreplaceability and the existence of insurmountable ethical limits, cases in which harm cannot be ethically justified, regardless of potential scientific benefits. Proposals exceeding these limits should be rejected outright. Furthermore, committees should encourage researchers to engage in ethical reflection through initiatives such as ARRIVE and PREPARE guidelines, while also promoting the acceptance of humane endpoints in regulatory frameworks. Researchers should actively push for the recognition of alternative methods and question regulatory inertia that still mandates lethal experiments even when death is predictable through validated scientific models. Beyond institutional efforts, public and scientific awareness must be raised. Writing letters to editors, engaging media channels, and fostering open scientific discussions can help expose unnecessary suffering in research and strengthen the movement toward more humane practices. Ethical scrutiny should not be limited to proposal review; it should extend to the post-publication stage, critically assessing whether published studies align with contemporary ethical and scientific standards. Finally, we explore how to initiate prohibition processes for highly invasive procedures by institutionalizing upper limits of harm as inviolable ethical boundaries. While some countries have banned extreme procedures, these measures remain fragmented and inconsistently enforced. Establishing a global ethical threshold for animal research, where scientific benefit can never justify certain levels of suffering, should be a priority. This presentation aims to contribute to this paradigm shift, reinforcing the need to eliminate ethically indefensible experiments and consolidate prohibitive advances in research policies.

Presentation: Oral

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Challenges in using clinical data for identification and classification of respiratory sensitizers

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Respiratory sensitization can cause severe conditions such as allergic asthma and allergic rhinitis. It is a two-step process: initial exposure to a substance primes the immune system, and subsequent exposure triggers an immunologically mediated hypersensitivity reaction. This distinguishes “true” sensitizers from respiratory irritants. While low molecular weight chemicals (LMWCs) are known to cause respiratory sensitization, primarily in occupational settings, exposures can also occur outside the workplace. Despite increasing research, there are no widely accepted *in vitro*, *in silico*, or *in vivo* models to accurately predict LMWC respiratory sensitizers. As a result, regulatory decisions rely heavily on human clinical data, which presents significant limitations. Although clinical and regulatory frameworks share the goal of protecting human health, their approaches differ significantly. Regulators focus on the chemical classification for risk management. Under the UN Globally Harmonized System (GHS), a substance can be classified as a respiratory sensitizer without demonstrating an immunological mechanism, even though a separate hazard class exists for respiratory irritants, which can cause similar symptoms. This regulatory ambiguity complicates the accurate classification of LMWCs. Clinicians prioritize diagnosis and treatment, leading to diagnostic methods and reporting practices that often lack the rigor needed to establish causation or investigate underlying mechanisms. Among available methods, the specific inhalation challenge (SIC) test is widely used but has critical limitations. Current regulations should restrict the classification of respiratory sensitizers to chemicals with clear immunological mechanisms. Given the absence of validated test models, regulatory reliance on human clinical data remains problematic. Due to their limitations, SIC tests cannot serve as the sole basis for classification, as currently allowed. They should be part of a comprehensive weight-of-evidence (WoE) assessment to ensure scientifically sound hazard classification and prevent misclassifications. This presentation will review findings from the ECETOC Task Force, evaluating various clinical methods and data sources and their implications for hazard identification and classification from a scientific perspective.

Presentation: Oral



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Challenges and gaps in the regulation of animal research in Latin America

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The regulatory landscape of animal experimentation in Latin America presents significant challenges, particularly in oversight and enforcement. In countries such as Chile, Colombia, Peru, Argentina, and Mexico, existing frameworks often lack effective control mechanisms, placing much of the responsibility for compliance on the institutions conducting the research. These gaps raise concerns about animal welfare standards across the region. Of the countries reviewed, all require the establishment of institutional bioethics committees in facilities conducting animal research, however, only Mexico and Argentina have centralized registries for animal breeding and maintenance facilities, with Mexico being the only country that demands reporting on the species and number of animals used. Consequently, there is no official number of animals used for research in Latin America.

By the lack of clear regulation policies, scientific communities across the region have self-regulated through professional associations and compliance to international standards, such as those from the United States, the European Union, and international scientific associations. International research funding requirements also drive institutions to align with higher standards, however, this presents a challenge, as access is not universal. The lack of centralized registries complicates the efforts to ensure ethical animal research practices across the region.

In Chile, the Animal Welfare Law lacks guidelines for laboratory animal experimentation and only addresses it in general terms. The law creates a National Animal Bioethics Committee, indicating that it should provide guidelines for experimentation, however, it fails to clearly define the Committee's attributions, whether its recommendations will be binding, or if it has enforcement authority. As a result, no national regulation for animal experimentation has been published since the law came into force. While Colombia has Resolution 8430 of 1993, as well as a Research Ethics, Bioethics, and Scientific Integrity Policy, and explicitly mentions the 3Rs principle in its national regulations, it refers to foreign guidelines and still faces challenges regarding the use of animals, particularly in terms of local context adaptation, oversight, and record. In Peru, the Animal Protection and Welfare Law provides a general framework, restricting the use of animals in testing to cases where results cannot be obtained through alternative methods. However, the lack of specific regulations and effective control mechanisms challenges its enforcement and leads to a degree of subjectivity in the assessment of alternatives to animal use. In Argentina, the Animal Protection Law prohibits acts involving mistreatment or cruelty to animals, such as mutilation and experimentation beyond what is indispensable, but lacks specific regulation besides ANMAT's guidelines for animal facilities. Mexico, despite having NOM-062-ZOO-1999, faces serious challenges in enforcement, considering the difficulty in monitoring facilities that conduct experimentation. It is debated whether the records are complete and whether the categories under the institutions are registered are accurate.

The regulatory gaps in Latin America highlight the need for legislation that aligns with the evolving knowledge about animal welfare, promoting a shift towards transparency and ethical responsibility in animal research in the region.

Presentation: Oral

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The RAT list: Tracking replaceable animal tests and identifying systemic barriers to non-animal methods

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Over the past 30 years, there have been significant developments in the replacement of tests on animals, particularly for regulatory purposes. Non-animal methods have been developed that can replace wholly, or in part, a number of animal tests across multiple product sectors. However, while it may be commonly assumed that animals are not used, or only very rarely, in cases where non-animal methods are available, the reality is that such tests can persist, and even increase, long after non-animal approaches have been established. This paradox is a key part of the bigger picture of why, despite the availability of non-animal methods, animal tests continue to be used. The reasons include scientific, regulatory, practical and even psychological factors that hinder the widespread adoption of non-animal methods.

This presentation will focus on ten specific regulatory tests that are still performed on animals, despite the availability of non-animal approaches that have been shown to produce reliable and scientifically valid results. These tests include rabbit pyrogen, skin and eye irritation, skin sensitization, antibody production, and batch potency and safety tests. Collectively, these ten tests result in an estimated 1.2 million animals being used annually in Europe, according to publicly available data from 2022. This persists even though the EU has legal requirements for the adoption of non-animal methods where feasible. In regions without such legal mandates, the extent of animal use is likely even higher.

The Replace Animal Tests (RAT) list, created by Cruelty Free International, tracks these ten tests and compares their use across different regions including Europe, North America, South America and Asia. It provides clear evidence of their persistence and highlights the key factors inhibiting the widespread uptake of non-animal methods, including scientific, regulatory, practical and psychological barriers.

In this talk, we will explore these inhibiting factors and discuss strategies to overcome them, including increased public and political pressure, global harmonization of data requirements, and timely updates to legislation. Key actions include clearer regulatory guidance, better enforcement, and enhanced communication between regulatory authorities and companies. Advancing the transition to non-animal methods will also require greater collaboration, dedicated funding, and shifts in scientific perspective. By addressing these challenges, we aim to accelerate the widespread adoption of non-animal methods, particularly for the ten tests on the RAT list, which have readily available non-animal replacements, ultimately improving both human and animal health outcomes.

Presentation: Oral



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Have the non-technical summaries of animal experiments in Europe improved? An update

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Following a review of Directive 2010/63/EU on the protection of animals used for scientific purposes in the European Union (EU), non-technical project summaries (NTS) of all approved projects must be published in a central database using a standard template. Our initial review of the NTS, reported in ALTEX in 2018 [1], found significant deficiencies in their accessibility and quality, particularly in the “adverse effects” section where the harms to the animals were not adequately described. These findings, alongside feedback from other stakeholders, contributed to legislative changes, including the establishment of the EU ALURES central database [2] and the adoption of a revised NTS template.

This talk will present the results of an updated review to assess if these legislative changes have improved the accessibility and quality of the NTS. As in our previous work, we focus on NTS from the Germany and the United Kingdom (UK); although the UK is no longer part of the EU, it continues to use the same template. We found significant improvement in the reporting of five of the six essential elements of the “predicted harms” section. However, there was no significant improvement in the reporting of adverse effects, with only 41% of German NTS and 48% of UK NTS fully reporting this critical element.

While progress has been made, researchers still appear to struggle with describing the impact of their research on animals. Unless these gaps are addressed, the potential of NTS to support the 3Rs and evidence-based policymaking will remain limited. The ongoing deficiencies in the “adverse effects” section highlight the need for further refinement in reporting practices. To address this, we have provided a checklist for competent authorities to ensure NTS are completed properly, along with a list of suggested terminology for standard administration and sampling procedures. We believe these measures will help ensure NTS fulfill their purpose of promoting transparency and encouraging the sharing of best practices in the 3Rs, and we encourage collaboration between competent authorities and researchers to facilitate this.

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Presentation: Oral

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A viable replacement for animal models of epilepsy: Combining human brain data and computational modelling

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Epilepsy is a debilitating brain disease that affects around 2-4% of people worldwide. The disease is characterized by unprovoked and recurring seizures (abnormal electrical brain activity), which are patient specific and extremely difficult to predict. Although many patients can achieve some seizure control with current medications, about one third of patients with epilepsy do not respond adequately to medication and are far more challenging to treat.

The overwhelming majority of neuroscience research in epilepsy uses animal experiments. Animal models (typically mice & rats) are used to test hypotheses by artificially inducing epilepsy via drugs, invasive electrical stimulation, and genetic modification to produce seizures. Although there is a plethora of data generated, there is very little understanding of the underlying physiological mechanisms. It is controversial whether seizure-like behavior generated from animal models is even comparable to that found in a human brain *in vivo*.

We propose a combination of voluntary human data and computational modelling as a viable alternative to animal models of epilepsy. Specifically, we use photosensitive epilepsy in humans where a seizure-like response is triggered by a non-invasive sensory stimulation (strobing light source) and captured using electroencephalography (EEG). This ubiquitous procedure is quite safe and routinely performed for epilepsy diagnosis. We have performed this experiment with a modified protocol in humans that produces (harmless) epileptic responses that are electrographic seizures. These responses to sensory neurostimulation are then mathematically analyzed to gain clinically relevant insights into the nature of seizure transitions in humans. This is performed without invasive techniques such as electro-stimulation, chemical induction or genetic modification as used in animal models and is such endogenous. Within this novel framework we are able to examine changes in brain state before a seizure transition, as well as quantify the individual variability within and between epileptic patients and patient groups. Our methodology also unambiguously circumvents the significant problems associated with the differences between animal and human brains. Our novel multidisciplinary approach moves away from traditional neuroscience research paradigms using animal experimentation and aims to minimize and ultimately replace animal models with computational modelling of non-invasive human data.

Presentation: Oral



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Replacing animal models by using *in silico* computational fluid dynamics, *ex vivo* blood circulation loops, and *in vitro* cardiovascular simulators to evaluate cardiovascular devices

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Cardiovascular and respiratory diseases are the leading causes of death worldwide. Patients suffering severe cardiac or cardiorespiratory failure may require implantation of devices such as stents, heart failures, ventricular assist devices (VADs), extracorporeal membrane oxygenation (ECMO), or total artificial hearts (TAH). Optimal device designs and operation strategies are vital in ensuring best-possible patient outcomes. The medical device development pathway often requires extensive animal experimentation to develop and enhance the performance of these devices. Our research focuses on the development of high-fidelity computational fluid dynamics (CFD) simulations, physiologically representative mock circulatory loops (MCLs), and *ex vivo* blood circulation loops (BCLs) to reduce or replace the need for animal studies.

Early-stage device development and evaluation can occur using CFD simulations; computer simulations of blood flow through devices and the vasculature. Our sophisticated models have been developed to simulate blood clotting (thrombosis – via bulk flow dynamics and biochemistry models) and blood cell rupture (hemolysis) which we have used to optimize devices prior to animal experimentation.

Physical device evaluation can occur using MCLs to investigate their hemodynamic performance in a realistic benchtop simulator of a patient. Our world-class models include the four heart chambers with Starling-responsive ventricles and atria while the vasculature is modelled in systemic and pulmonary circulations with arterial and venous compliance and resistance, alongside biphasic and autoregulatory coronary and cerebral circulations. Almost any cardiovascular device can be simply inserted into the MCL prior to extensive and rapid characterization and ultimately optimization.

Hemocompatibility can be evaluated using benchtop BCLs which mimic a small-volume replica of a patient with a device inserted. Fresh whole human blood is circulated around the BCL and through the device to compare blood compatibility with a clinical standard. This system allows for high resolution assessment of a device's potential to clot or damage blood cells including markers such as hemolysis, platelet activation, cell counts, and von-Willebrand Factor degradation.

Overall, our use of CFD, *in vitro* MCLs, and *ex vivo* BCLs have provided an extensive and reliable suite of technologies to reduce and replace the need for animal models when developing cardiovascular devices. The outcomes from these platforms demonstrate the value of cardiovascular animal replacement models towards improving clinical practice and device optimization, and ultimately, achieving better patient outcomes.

Presentation: Oral

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Developmental neurotoxicity of bisphenol A and its alternative chemicals in human stem cells and its extrapolation to ecological species based on cross-species extrapolation

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This study presents an alternative assessment strategy for the developmental neurotoxicity of bisphenol compounds (BPA, BPF, and BPS) through a novel cross-species extrapolation framework that bridges human toxicology and ecotoxicology. Current risk assessment practices typically require separate testing regimens for human health and ecological impacts, resulting in increased animal use and regulatory inefficiency. We demonstrate a more integrated and efficient approach that leverages human stem cell data as a foundation for cross-species predictions.

Our methodology combines *in vitro* human embryonic and neural stem cell assays with Bayesian network (BN) modeling to quantify relationships between key events in the adverse outcome pathway, followed by cross-species extrapolation using Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) and Genes to Pathways-Species Conservation Analysis (G2P-SCAN).

BN analysis revealed strong correlations between molecular events, with increased brain-derived neurotrophic factor (BDNF) levels strongly associated with decreased cell viability. Our novel protein prioritization strategy first identified densely connected protein complexes containing bisphenol targets using Molecular Complex Detection algorithm, then evaluated pathway conservation across species. This approach revealed significant conservation of toxicity pathways across species, with human genes showing greater than 50% similarity to orthologs in ecological model organisms. Proteins involved in NMDA receptor binding and glutamate homeostasis displayed extensive conservation with over 500 identified orthologs spanning diverse taxa.

This alternative assessment strategy exemplifies how human *in vitro* data can be leveraged to predict ecological impacts without extensive animal testing, while providing mechanistic insights applicable across species boundaries. Our approach offers a resource-efficient path forward for integrated chemical risk assessment that aligns with the One Health perspective while addressing 3Rs principles in toxicology testing.

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Presentation: Oral



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A “serious game” to train ethical committee members in NAMs

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The TATABox (Towards Alternatives To Animal Testing-box) is an innovative board game created in 2022 by Altertox, a Belgian company co-founded by French toxicologist François Busquet. The TATABox offers a first format for toxicology and a second format for study design dedicated to the ethics committee. Dedicated to facilitating the transition to animal-free research methods, this game aims to understand the various stages of design, development, and validation of alternative methods and to clearly identify all the stakeholders involved. It also promotes discussions on scientific mediation and communication. By focusing on toxicology, the TATABox allows participants to understand the NAM ecosystem. A new version, integrating the role of ethical committee members, was developed with two new scenarios to be used in the initial and continuing training of designers. First, “co-creation”: the objective of the study is determined by the participant, with direct support from other participants acting as ethical committee members. The ethical committee members intervene throughout the game by adding tiles from the TATABox to integrate the principles of the 3Rs when fit for purpose. Second, “the design of the study”: the participants use the tiles from the TATABox to build a study without the intervention of the ethical committee members, which only gives its opinion at the end of the game.

Presentation: Oral

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Indian Society for Alternatives to Animal Experiments – Genesis and update

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Alternatives to animal experiments, later the NAMs, have become a serious topic among the academic, scientific and regulatory communities in India since the establishment of the Mahatma Gandhi-Doerenkamp Centre (MGDC) for Alternatives to Animal Experiments and the Gandhi-Gruber-Doerenkamp Chair for Alternatives by the Doerenkamp-Zbinden Foundation (DZF) at Bharathidasan University, Tiruchirappalli, India, in 2009 [1,2]. The Centre and the Chair have been engaged in overwhelming work through research, hands-on training workshops, seminars, conferences, etc., especially through national and international collaborations. The pinnacles of glory have been i) the decision of the University Grants Commission (UGC) to ban the dissection of animals as an aspect of Biology education, ii) the Medical and Pharmacy Councils (MCI and PCI) of India prescribing computer-based learning of animal experiments, iii) the decision of DCGI / CDSCO to ban animal testing of cosmetic finished products and ingredients, and iv) amendment to the New Drugs and Clinical Trial Rules (2023), aiming at replacement of use of animals in research, especially in drug testing, to mention a few. Subsequently, MGDC came to be supported by the UGC for 5 years, by the “National Centre for Alternatives to Animal Experiments (NCAAE)”. Thus, the alternative movement in India is going strong to reach newer heights. To strengthen this movement for propagation, popularization, prescription/adoption, and implementation of alternative methods (NAMs) among different stakeholders, the Society for Alternatives to Animal Experiments was founded in 2018, with HQ at NCAAE, Bharathidasan University, Tiruchirappalli. The Society was launched at the First National Conference for Alternatives to Animal Experiments (NCAAE) held at Jamia Hamdard (Deemed University), New Delhi, on Nov 27, 2018. The Society has been registered and inaugurated. Annual conferences were organized at i) National Facility for Biopharmaceuticals, Mumbai (2019); Bharathidasan University (2020); DPSRU, New Delhi (2021); University of Kerala, Thiruvananthapuram (2022); and Aligarh Muslim University, Aligarh (2023). The 2024 conference was the 4th Asia Congress, with speakers from Japan, Korea, China, Sri Lanka, the USA, Austria, Slovenia and England. Pre- and post-conference workshops always accompany the conferences on different themes. The Society is in MoU with JSAAE, EUSAAT, ESTIV and ASCCT. The Society is now one of the founding members of the Asian Federation of Societies for Alternatives to Animal Experiments (AFSAAE), with the other members being Japanese, Korean, and Chinese Societies. While each federating Society shall operate within the respective countries to bring about gross changes in animal use in experimentation, all 4 societies expect to form a strong network to share expertise and development.

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Presentation: Oral



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How *in silico* approaches can increase confidence in environmental hazards identification to address global pollinators decline and to support environmental risk assessment policy

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The new EU-funded project WildPosh (<https://wildposh.eu/>) was launched in early 2024. As part of the EU pollinators initiative, the project has an ambition to provide a better understanding of the routes of exposure of wild pollinators to chemicals, to investigate toxicological and ecological effects on terrestrial biodiversity and to support the environmental risk assessment policy.

The Istituto Superiore di Sanità (ISS), Rome, Italy is leading the work on *in silico* modelling activities within the project with the purpose of improving the existing *in silico* prediction methodologies for toxicity endpoints relevant to pollinators. The work includes a critical review of all existing relevant prediction methodologies, such as (Q)SAR models, category approach or read-across; identification of the most important gaps and sources of uncertainties and refinement of *in silico* predictions using existing data and data that will be generated within the project.

Evaluation of regulatory readiness of (Q)SARs will be performed according to the OECD QSAR assessment framework (QAF) and to the specific requirements of the most important regulatory agencies. The results will be used, together with other data produced by the project, for development of the WildPosh Toolbox, an integrated system-based risk assessment tool with the global goal of reversing pollinator population decline and reducing the harmful impacts of pesticides and other chemicals.

Presentation: Oral

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Can a human blood waste product replace animal blood in hemocompatibility assessment of medical devices?

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Mechanical Circulatory Support (MCS) supplements or completely replaces the functions of the heart and/or lungs and may be used acutely to assist medical procedures (e.g., valve repair) or chronically in those

with organ failure. Current approaches to regulatory approval of such technologies involve extensive pre-clinical evaluation of the safety and efficacy of novel devices, especially for implantable devices such as total artificial hearts. The consequences of poor hemocompatibility and biocompatibility account for the majority of fatal complications. Hemocompatibility assessment of MCS and ECLS components is currently guided by, among others, ISO 10993-4 and ASTM F1841, which provide the approaches to evaluation practices and reporting requirements. In prior standards, it was identified that human blood was preferable for use in testing, although recent revisions acknowledge that while blood sources may differ in blood trauma sensitivity, the donor source should simply be recorded; sourcing blood from humans is no longer recommended. International groups cite a preference for using large mammal donors for various pragmatic reasons: i) in some countries, access to human blood is highly regulated and limited; ii) the volume of blood required (typically > 800 mL) is too large for a single human donor, and; iii) ethical concerns given human blood is a waste of a life-giving resource. To overcome these limitations, we have explored the opportunity to use a waste product – venesections from patients with haemochromatosis – to replace the use of animal blood in the pre-clinical assessment of MCS devices.

Haemochromatosis is a hereditary iron overload disorder that affects up to one in two hundred individuals of European descent. Excessive iron within the body results in various complications that could be easily managed: regular bloodletting (venesections) of up to 900 mL directly removes iron from the organism, while the depleted blood cell levels stimulate iron-intensive processes such as erythropoiesis. While it is ethically impermissible to take such large samples from healthy individuals, the venesections in haemochromatosis are a therapeutic necessity. We can utilize this, provided the blood has unchanged hemocompatibility characteristics. We have demonstrated that upon first diagnosis, where blood iron levels are high, the cells in venesections are rigid and excessively cluster together (aggregate) and thus do not reflect blood from healthy donors. However, we also observe that upon successive venesections, cell rigidity returns to levels typical of normal health and the tendency for cells to aggregate significantly decreases. While the blood collected from those who are newly diagnosed with haemochromatosis may not be suitable for pre-clinical assessment of medical devices, blood from those with well-maintained iron levels represents a valuable resource to replace animal products. Indeed, we demonstrated that the mechanical sensitivity of blood cells from those with regular venesections reflects those of normal healthy donors.

These results will be presented in concert with new findings from pre-clinical evaluation testing of clinically-approved MCS devices. Discussion will focus on the validity of using human blood in pre-clinical assessment of MCS, and the opportunity to influence relevant international standards to promote the replacement of animal blood with a human waste product.

Presentation: Oral



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Multifunctional hydrogels decorated with bioactive hydroxyapatites to develop 3D-printed solution aiding wound disinfection and tissue regeneration

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Chronic wounds are a major healthcare challenge, affecting the quality of life of millions of people. It is widely recognized that traditional wound care methods are often inadequate to manage the complexities of chronic wounds. Tissue engineering has been explored as an alternative to conventional wound management in clinical practice, and the use of biomaterials as skin substitutes has emerged as a promising solution. Indeed, a wound dressing must perform multiple functions, protecting the injury without being impermeable and maintaining a moist environment required for effective healing.

In this study, 3D printing technique was used to develop a by-layered hybrid patch that may be useful for wound management and as a scaffold to facilitate the engineering of 3D skin models for the discovery of new treatments. This approach adopted circular economy principles by using waste raw materials, such as gelatin and chitosan, properly combined to create a 3D printable formulation. These biopolymers, after printing and freeze-drying, form highly porous structures that promote permeation and proliferation of the cells within. Additionally, a bioactive hydroxyapatite phase (HA) with low crystallinity and doped with

antimicrobial ions is incorporated, which has multiple functions. It not only helps to modulate the ink's viscosity but also improves bioactivity and can provide long-term antimicrobial activity.

By leveraging advanced 3D printing techniques, two distinct layers were created, each differing in composition and morphology. This variation in the layers allows for tailored properties, enhancing the ink's ability to mimic natural tissue and promote optimal cellular interactions. The outer and more compact layer, designed to replicate the epidermis, while the inner, porous layer, akin to the dermal tissue, is highly biocompatible, bioresorbable, able to absorb wound exudates and to provide regenerative stimuli and long-term antimicrobial activity, thanks to chitosan properties and to ions hosted in the HA lattice. Finally, to boost their antimicrobial potential, the inner layer can also be medicated by adding antimicrobial drugs to address the patient's need. These patches have been extensively characterized to evaluate their morphology, porosity, moisture permeability, fluid absorption capability, and degradation stability. Additionally, both short-term and long-term antibacterial activities have been assessed *in vitro*. The biocompatibility, bioactivity, and regenerative effects of these biomaterials were further validated through *in vivo* studies with the well-established invertebrate model, *H. verbana* (medicinal leech). The patches were extensively colonized by cells and integrated well into the tissues of leeches subjected to controlled wounding.

In conclusion, the developed hybrid matrices, inspired by the natural composition and structure of the skin, have been shown to be multifunctional, combining sustained and targeted antimicrobial activity with optimal biocompatibility properties, favoring cell adhesion and proliferation, and with regenerative capacity. They exhibit excellent physicochemical properties, including exudate absorption, moisture permeability, biodegradability and effective drug release kinetics. All this evidence demonstrates that the designed devices can have multiple functions, both as medical devices for wound management and as porous matrices for engineering 3D models *in vitro*.

Presentation: Oral



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Composite injectable formulations for 3D printing of biomimetic bone matrices: Customized tools for the bio-engineering of organotypic bone models

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Due to the increasing needs for 3D constructs for tissue engineering (TE) approaches, the 3D-printing process has attracted even more interest to better fulfil the TE demands and to improve the model fidelity in reproducing the complexity of biological architecture. In the common hope, 3D-printed tissues will overcome many of the shortcomings of traditional tissue engineering ensuring precise control over structural design, cells response, and cell-laden in 3D-constructs. However, advancement of bio-printing technology is limited by the availability of materials facilitating bio-printing process as well as cell viability and providing of tissue-specific cues.

In this contest, our research addresses the challenging task of formulating nanocomposite bioinks for the development of biomatrices recapitulating the bone ECM microenvironment capable of promoting regeneration processes *in vivo* and useful as porous scaffold for the design of 3D *in vitro* models aimed at the study of diseases-related biological mechanisms and the discovery of precision drug therapies; in both cases, a high model fidelity with the tissue is significant for successful application and study.

Bioinks components must be chosen based on biocompatibility, biochemical signals, crosslinking mechanisms, degradation, and printability that should ensure maximum viability and minimum stress to the cells possibly encapsulated in the matrix, before, during and after printing. To face this challenge, nanocomposite inks have caught our attention because the incorporation of nanoscale materials has allowed for significant tunability of the mechanical, rheological, structural, and biological properties of inks and printed constructs. The modulation of particles configurations in terms of composition, surface chemistry, shape, size, concentration, make nanoparticles of high interest as bioink additives for the improved design of bioactive constructs for tissue engineering.

Biomimetic synthesis approaches were exploited to engineer hydroxyapatite nanoparticles (nHAs) functioning as bioactive ingredient into printable inks and bioinks for bone tissue engineering applications and the effect of their incorporation in different polymeric matrices was investigated. Two engineered nHAs were used to prepare different ink formulations, one incorporating mesenchymal stem cells in a nanocomposite bioink composed of methacrylate-gelatin, photo-polymerizable with Vis-light, and enriched with Mg-CO₃-doped-nHAs to evaluate their effects on the bio-printing process and outcomes on cells. The second, an acellularized-inks whose rheology was modulated to avoid crosslinking steps, allows to obtain freeze-dried scaffold highlighting a multi-dimensional porous structure, stable for prolonged time and suitable for TE in perfusion bioreactors for long time points.

In both studies nHAs demonstrate to enhance the printability of inks and the stability of the 3D structures representing a valid strategic tool for the development of printable bioactive inks, allowing a good model fidelity in the engineering of cells-loaded or not 3D-biomatrices. This approach enables the 3D printing of composite bio-constructs highly mimetic of the bone ECM promising for 3D disease modeling and drug screening applications, and address standardization and scalability, which are among the most common challenges in developing organotypic bone models, useful to avoid the use of animal models.

Presentation: Oral



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Multiparametric screening of MASH clinical candidates in human liver spheroids predicts clinical outcomes and reveals potential novel combinations for anti-steatotic and anti-fibrotic therapies

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Background and aims: Metabolic dysfunction-associated steatohepatitis (MASH) is a progressive severe disease characterized by hepatic lipid accumulation, inflammation, and fibrosis which lacks any approved drug therapy. Novel approaches to identify therapeutic candidates that predict clinical responses are needed. Human pre-clinical models, including organoids/spheroids, are powerful models for drug testing and discovery. Pre-clinical models combining fast, robust, high-throughput compatible, and predictive outputs are still a limiting factor in identifying novel clinical therapies. We performed a comparison of 25 existing clinical candidates and controls covering different mechanisms of actions and modalities for MASH using a human-derived 3D spheroid model.

Methods: We used a human-derived 3D spheroid model, containing primary human hepatocytes, Kupffer cells, liver endothelial cells, and stellate cells from healthy donors to induce a MASH phenotype. We screened simultaneously for the toxicity and MASH efficacy of 25 current clinical candidates in a dose-range manner. We selected secreted lactate dehydrogenase (LDH), intracellular triglycerides (TGs) and secreted pro-collagen 1 (PC-1) as surrogate markers of cytotoxicity, steatosis, and fibrosis. LDH was measured at days 3, 5, 7 and 10, TGs at day 10, and PC-1 at days 7 and 10.

Results: Incubation with a MASH-inducing cocktail for 10 days recapitulates the main hallmarks of MASH including steatosis, and fibrosis by a 10-fold increase of triglyceride levels and PC-1 secretion compared to healthy control respectively. The clinical candidates comprehend FXR agonists, lipid synthesis inhibitors, THRbeta agonists, PPAR agonists, or anti-fibrotic compounds.

We reveal a distinctive resolution of MASH hallmarks from different anti-metabolic drugs.

The DGAT2 inhibitor Ervogastat, ACC inhibitor Firsocostat, and FASN inhibitor TVB-2640 compounds showed a 25.3% (p-value < 0.01), 35% (p-value < 0.0001) and 23.1% (p-value < 0.05) decrease of triglyceride content versus healthy control respectively. However, only Ervogastat showed an additional anti-fibrotic profile with a 24.8% reduction PC-1 at 6.3 micromolar (p-value < 0.05). On the other hand, the anti-fibrotic alpha V, beta 1 integrin inhibitor PLN-1474 showed a strong reduction of 65% reduction of PC-1 at 6 micromolar (p-value < 0.0001).

Conclusion: We show a robust screening platform for MASH drug candidate testing and identify several anti-metabolic and anti-fibrotic hits. DGAT2 inhibition results in a concomitant improvement of steatosis and fibrosis in a human liver spheroid model. This data shows that suppressing steatosis would result in fibrosis prevention. We reveal ACC or FASN targets as potential good drug combination approaches with anti-fibrotic drugs such as integrin inhibitors.

Presentation: Oral

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Use of CATMoS in driving non-animal approaches in EU regulations

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Acute Oral Toxicity (AOT) testing is crucial for chemical safety assessment in the EU and globally. While regulations mandate AOT evaluation, they also require animal testing only as a last resort (Art. 25 REACH). Although not extensively defined, the “last-resort” requirement implies *in vivo* testing should occur only when necessary for safety decisions and no suitable non-animal methods (NAM) are available. This necessitates a transparent evaluation of available human-relevant approaches before resorting to animal testing. However, inconsistent implementation highlights the need for clearer guidance and stronger regulatory accountability.

Building on AFSA Collaboration’s analysis (PMID: 38142814) and its Regulatory Transformation Strategy, this project aims to enhance compliance with the last-resort requirement. Given the ethical and regulatory urgency of replacing animal testing, AOT testing under REACH and CLP serves as a case study to set a regulatory precedent.

Following a regulatory and scientific readiness analysis of existing technologies, the project has established a decision tree framework to:

- Improve regulatory guidance and transparency in “last-resort” implementation;
- Ensure consistent application of NAMs where scientifically valid and regulatory suitable;
- Address gaps for application in REACH and CLP regulations.

The framework integrates waiving principles and NAM implementation to ensure regulatory suitability. CATMoS is a leading example of a ready-to-implement NAM for AOT assessment, recognized for its high performance and regulatory relevance.

The analysis finally highlights key actions to accelerate NAM adoption:

- Refinement of guidance documents, incorporating decision tree frameworks, to improve transparency and consistency in decision-making and legal certainty;
- Increased funding for confidence-building initiatives in NAMs, such as CATMoS, among industry stakeholders and regulators and promotion of international collaboration and harmonization;
- Expanded research into mechanism-based approaches to enhance predictive toxicology and address gaps in the applicability domain.

By enhancing accountability and NAM integration, this project aims to accelerate the shift to non-animal assessments, setting a precedent for broader regulatory adoption.

Presentation: Oral



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Advancing education on animal-free safety assessment – A global perspective

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Education is one of the pillars for advancing the promotion and uptake of animal-free safety assessment for chemicals. Global progress in animal-free science for both human health and the environment is becoming increasingly important, due to expansion of chemicals legislation across the world which is creating an urgent need to progress the adoption and use of non-animal New Approach Methodologies (NAMs) around the world.

In particular, the EU and UK legislative frameworks for chemicals are under review, and a roadmap to phase out animal testing for chemicals safety assessment is under development. Therefore, ramping up education programs to upskill both industry and regulators is vital to achieve the objective of the roadmap and ensure that animal-free safety assessment is integrated into EU and UK chemicals legislation. This session provides an overview of training and educational initiatives available in the EU and UK on animal-free science for chemicals safety assessment, addressed to industry and regulatory scientists to enable them to embrace new approaches and give toxicologists the reassurance they need that data from NAMs are robust and reliable.

Presentation: Oral

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Accelerating the development of complex *in vitro* models (CIVMs) for rare disease therapeutics in India

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In India, a rare disease is defined as a condition that affects not more than 500,000 persons under the New Drugs and Clinical Trial Rules (2019). Currently, about 72-96 million people in India live with a rare disease. While around 450 rare diseases have been identified in India, but the therapeutics approved by the Drugs Controller General of India (DCGI) are available only for about 12-15 of them. This highlights the critical need for availability of effective and affordable therapeutics. Recent studies show that the use of organ-chips can lead to a reduction of 10-26% of research and development costs; and increase of additional \$3 billion annually for the pharmaceutical industry. This could help to reduce treatment costs and increase access to rare disease treatments, especially for low and middle-income countries, such as India. In this talk, I would cover the rare diseases of concern in India and their prevalence; and status quo of rare disease research in India that employs CIVMs, including induced pluripotent stem cells (iPSCs), organoids, organ-on-chip, etc. We recently published a review on the use of CIVMs for preclinical and clinical stages and discussed possible actions to enable their use in developed as well as middle-and low-income countries. The talk would also cover recommendations on how the use of CIVMs can be further enabled to propel rare disease therapeutics in India and identify actionable stakeholders who could support these recommendations. Fostering indigenization of technologies and reducing the reliance on global supply chains would be critical to make these technologies affordable in India. India has established 12 rare disease Centers of Excellence – incorporating funding and dedicated research verticals for CIVMs in these centers could enable the acceleration of these methods in India. Increasing the awareness of potential of CIVMs and involving the patient associations in research further ensures alignment with patient needs, fosters trust, and increases the impact of research outcomes. Thus, via this session, we hope to initiate discussions on policy directions that can enable this research in India and gain insights from established ecosystems in the global north and developing ecosystems in other global south countries, such as Brazil.

Presentation: Oral



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A problem formulation from scratch: What could an exposure-led NGRA look like for the farmer's toolbox products

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The Next Generation Risk Assessment (NGRA) concept is an exposure-led, tiered assessment framework originally developed for cosmetic ingredients and designed to allow safety assessment with no use of animals testing. Its applicability in other industries is being assessed and the applicability in the context of crop production and protection is reviewed within this presentation. For the exposure part of the equation, an overview of human and environmental external exposure routes for conventional and biological active ingredients and their metabolite will be reviewed, considering dietary and non-dietary routes of exposure. Depending on the jurisdiction different deterministic models are currently in use. However, the use of internal exposure modeling is currently very limited and the shift to internal exposure is essential to implementing next generation risk assessment. PBK models offer a great possibility to provide internal exposure estimates, and the presentation will review some case examples where this is possible both in an R&D or regulatory context related to crop protection. This exploration is also possible due to the richness of *in vivo* and *in vitro* kinetic and metabolism data generated in our sector.

Similarly, cases of points of departure based on toxicodynamic information (Mode of action studies for regulatory human relevance assessment, or more general bioactivity data for R&D purposes) will be reviewed.

Finally, the area of pesticide metabolite exposure provides an excellent opportunity for NGRA based read-across. Overall, NGRA is applicable in principle to safety assessment of crop protection products and their metabolism; some limitations in applicability are identified, first and most importantly lack of flexibility in regulatory frameworks which are currently based on traditional data requirements.

Presentation: Oral

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How to use human biomonitoring in risk assessment?

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While hazard assessment by new approach methods (NAM) is one column of next generation risk assessment (NGRA), also exposure assessment as the second column in risk assessment is an important tool, also in tiered testing strategies.

While there are many different tools for exposure assessment, including measurement of external exposure, e.g., via food, and modelling, human biomonitoring has attracted more and more attention in the past decade. The advantage of HBM is that internal aggregate exposure is monitored. A related disadvantage is that sources of exposure may be difficult to identify. Also, it has to be pointed out that exposure *per se* does not represent a risk and measured values must be correlated with reference levels based on toxicokinetic considerations and PBTK modelling, to conclude on whether or not a measured exposure is safe.

In this talk first results from a recently started biomonitoring study on apple farmers spraying plant protection products in Germany (aPPle study) are presented. In addition, the EU initiative PARC will be introduced that, in addition to work on NAM and NGRA also conducts biomonitoring studies.

Presentation: Oral



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NGRA in the pharmaceutical industry: Integrating *in vitro* data and physiologically-based kinetic (PBK) modeling for nephrotoxicity evaluation

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Traditionally a Permitted Daily Exposure (PDE) value is derived from NO(A)EL or LO(A)EL values obtained in sub-chronic and chronic animal studies. However, recognizing the need to implement new approach methodologies (NAMs) and uphold the 3R principles in risk management for cross-contamination in shared pharmaceutical facilities, this work employed an innovative approach for deriving the PDE value using vancomycin as a study case. We integrated *in vitro* toxicity data with physiologically based kinetic modeling-facilitated reverse dosimetry (PBK-RD) to identify and characterize the hazard of vancomycin-induced nephrotoxicity. The PBK-RD model was developed using PK-Sim version 11.3 in both rodents and humans, and its predictive performance was verified by comparing observed and simulated pharmacokinetic profiles. The vancomycin PBK model in healthy volunteers considered vancomycin physicochemical parameters, hepatic clearance and renal elimination mediated by glomerular filtration. The tissue-to-plasma partition coefficients and cellular permeability were calculated using the Rodgers and Rowland and the PK-Sim Standard equations,

respectively. The observed PK profile was within the 5th and 95th percentiles of predicted data, and the predicted PK parameters were within a 0.5- to 2-fold range compared to the observed data, being successfully validated according to OECD (2021) guideline. For the rodent PBPK models, the physicochemical and ADME parameters were identical to those in the human model, except for species-specific factors: fraction unbound, blood-to-plasma ratio, and hepatic and renal clearance. To improve the representation of vancomycin distribution within the kidney and its intracellular compartments, a parameter identification tool was used. Further, the rodent PBK model was back-extrapolated to humans. For accounting the vancomycin nephrotoxicity, four *in vitro* studies evaluating renal cell models from three species (human, rat and porcine), focusing on cell viability after 24 or 48 hours of incubation were analyzed. The BMD software version 3.3.2. from US Environmental Protection Agency (EPA) were used for Benchmark dose derivations. The 5% lower benchmark dose (BMDL5) was calculated by plotting the vancomycin predicted daily doses (mg/kg) against vancomycin nephrotoxicity response. The BMDL5 value of 0.01 mg/kg ($p = 0.524$) was chosen based on the lowest AIC value provided by the Exponential M5 model. In the next step, PBK-RD strategy was applied to choose vancomycin doses producing intracellular renal concentrations related with nephrotoxicity. The BMDL5 was selected as the PoD for the critical health effect, the PoD of 0.01 mg/kg was multiplied by the body weight of 50 kg, resulting in the PoD of 0.5 mg/day. Accounting for uncertainty factors (F), F1 and F2 were set to 1 due to the use of human data and the incorporation of interindividual variability within the model, respectively. F3 was set to 10 to address potential chronic exposure effects, while F4 and F5 were each set to 1, reflecting confidence in the dataset and the conservative nature of the PoD. This approach derived a PDE of 0.05 mg/day, lower than the traditionally calculated PDE (0.2 mg/day) based on the lowest clinical dose as the PoD. This study demonstrates the potential of PBPK-RD as a NAM-based strategy for refining toxicological assessments within the pharmaceutical industry.

Presentation: Oral



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GeneTox21 – A multiplexed *in vitro* platform for genotoxicity assessment

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Genetic damage is linked to a range of human diseases, and genetic toxicity testing is a cornerstone of regulatory toxicological assessments. Many traditional *in vitro* genetic toxicity tests are labor-intensive; New Approach Methods (NAMs) can facilitate efficient assessment of environmental chemicals while reducing reliance on experimental animals. Despite their advantages, many genotoxicity NAMs have not yet been adopted for regulatory purposes. GeneTox21 is an *in vitro* genotoxicity assessment platform that includes six higher-throughput NAMs (i.e., MicroFlow®, MultiFlow®, CometChip, TGx-DDI, MutaMouse FE1, and Ames II); it includes endpoints for mutagenicity, chromosomal abnormalities, and DNA damage signaling. The platform provides positive and negative classifications and mode of action determination; substance potency can readily be determined using the Benchmark Concentration (BMC) approach. Platform performance is being evaluated with 35 carefully selected reference compounds and 10 prioritized data-poor compounds; the current version integrates complementary results generated using the high-throughput ToxTracker® assay. GeneTox21 assessments, which have generated an incredibly rich dataset across 29 endpoints, indicate that the platform readily detects genotoxicants, including those that require metabolic activation. Analyses of selected reference compounds permitted resolution of instances where previously published results are contradictory (e.g., resorcinol and eugenol). Statistical analyses and mathematical modeling of the data indicate that only a subset of the GeneTox21 endpoints are required for accurate prediction of *in vivo* hazard. Specifically, Weight of Evidence (WoE) analysis indicates that the following assays are required for decisive, accurate prediction of genotoxic hazard: Ames II, ToxTracker® Blvrb, MultiFlow®, ToxTracker® Btg2, and Microflow®. Canonical discriminant analysis for quantitative prediction of *in vivo* hazard identified the same endpoints plus ToxTracker® Bsc12. Application of the GeneTox21 platform for the assessment of prioritized data-poor compounds demonstrated the importance of the integrated testing strategy for robust identification of genotoxic hazard; moreover, resolution of *in vitro* false positives. The Integrated Analysis Tool for Genotoxicity Assessment (IATGA) is a companion software tool that streamlines visualization, analysis, and interpretation of GeneTox21 data. In conjunction with this powerful tool, the GeneTox21 platform can examine genotoxicity profiles and potency ranking for data-poor compounds prioritized for regulatory assessment. Overall, the GeneTox21 platform provides comprehensive, multiplexed assessments of chemically induced genotoxicity. It is anticipated that the platform will contribute to the establishment and deployment of a NAMs-based system for chemical screening and assessment. In particular for cases where there are little or no experimental toxicity assessment data. Indeed, the platform is currently being integrated into a proposed Integrated Approach for Testing and Assessment (IATA) workflow.

Presentation: Oral

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Japanese initiatives in MPS utilization for ADME-Tox evaluation

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Microphysiological Systems (MPS) has attracted attention in human risk assessment of chemical substances. In Japan, a project for MPS development was initiated by AMED in 2017. AMED-MPS1/2, which aim at their industrial implementation, and MPS-RS1/2, which aim at their regulatory utilization, have been carried out since then. In this presentation, I will report on what is going on in these projects in Japan.

In assessing the effects of chemical substances on human health by new test methods, it is important to determine their “Context of Use (CoU)”. We focused on the evaluation of absorption in the small intestine and metabolism in the liver (first-pass effect), which are the first steps of the absorption of orally ingested chemical substances into the body. I will explain the outlines of the background why the first-pass effect was selected as CoU and discuss the specifications for the MPS and the cells used in the test system, which are guided by the establishment of the CoU. I will also discuss “Points to Consider” to meet these specifications and introduce examples of cell adhesion and oxygen supply that we are working on.

The discussions between MPS suppliers and MPS users are necessary to promote the practical application of these MPS, and we have launched the Japan MPS Initiative. I would like to introduce our activities in Japan and our collaboration with other countries.

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Presentation: Oral



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Computational perspective on NAMs: Data science and artificial intelligence supporting product design

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Physical products design is concerned with ingredients, formulations, quantities, sourcing, manufacturing and packaging processes, and foreseeing the path of the product along the lifecycle from design over production to the customers including logistics and marketing. Through complete specifications and digital twins, a lot of data has increasingly become available about all of these product design and lifecycle aspects. Exploiting that data for the benefit of reduced health hazards, improved environmental footprint and elimination of animal experiments can lead to safer and more sustainable designs of new products, but also greener redesigns of existing products. In addition to that, it also helps manufacturers to comply with new regulations around digital product passports, reports generation, and export opportunities.

This talk starts with an overview about the computational perspective related to product design data and algorithms, available data sources, and suitable data structures such as graphs and shards. The talk then proceeds to explain how such data could be organized and used to achieve this goal. For that matter, it introduces the concepts of linked data and large-scale distributed data processing and explains how confidential product design data can be linked up with open government data such as regulatory limits.

Moreover, it dives into subsequent graph mining, stealth computing and other confidential data science techniques to get insights and to produce actionable advice for decision makers. Those include product designers, customers and regulators, all of whom have a different perspective. Tolerance thresholds and exposure to knowledge differ, and customers are sometimes less concerned, sometimes more concerned than regulators about the implication of substances and effects. Hence, producing meaningful statements for those target groups while protecting business interests around confidential information remains a key challenge in this regard of designing a suitable computing platform. But likewise, the internal insights for the manufacturer need to be effectively collected and conveyed.

As unique contribution, the talk continues by proposing a novel platform architecture for scalable and confidential management of knowledge graphs around product design that has the potential to reduce the need for animal experiments. Any such reduction requires finding the necessary knowledge in previous data, which must be properly organized, aggregated and abstracted from previous studies. With emphasis on what is feasible from a computer science and computational science perspective, the current progress on such platforms as well as the unavoidable limits will be highlighted.

In the final part of the talk, the readiness of today's data science infrastructure for data-driven product design is discussed in conjunction with a view on available data in the health and environmental domains. This discussion encompasses an applied view on similar data-driven decision support systems and motivates to build a domain-specific product design platform with built-in data aggregation and exploitation capabilities.

Presentation: Oral

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Need of the hour: Balancing scientific objectives with practical knowledge

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Efforts to develop models to detect and understand the risks of potential respiratory sensitizers have been undertaken for many years and have intensified recently with the advancement of Integrated Approaches for Testing and Assessment (IATAs) and Defined Approaches (DAs) for dermal sensitization, as well as technological advancements permitting the development of complex *in vitro* and *in silico* models. Concordant to these efforts, practices for evaluating new methods and approaches have matured to provide a weight-of-evidence consideration of the advantages and limitations of any approach in the context of challenges as elucidated by other speakers, such as limited reference chemicals against which to benchmark the candidate approaches. Currently, within the Organization for Economic Cooperation and Development, a group of experts is working to draft a Detailed Review Paper to outline the regulatory needs and considerations, existing scientific knowledge, status of potential approaches which could be used within IATAs or DAs, and necessary next steps. It is envisioned that this effort will pave the way towards regulatory tools to advance the detection and characterization of potential respiratory sensitizers, meeting regulatory needs and advancing worker and public health protection.

Presentation: Oral



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The CHANGE project: Designing system-level interventions for bringing forward the date of effective use of NAMs

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The Collaboration to Harmonise the Assessment of Next Generation Evidence (CHANGE) is built upon the kind of interdisciplinary collaboration that risk and safety research relies on. CHANGE focuses on system factors as an area of research that could be further developed and customized for bringing forward the date of effective use of NAMs (Mathisen et al., 2024). By “system factors”, we mean potentially modifiable aspects of the organizational environment in which NAM-based research is conducted, interpreted, and acted on. The system factors, typically including technical and social factors (e.g., people, resources, culture, processes, policies, and legislation), may provide new opportunities for effective implementation of NAMs in regulatory toxicology.

The overall goal of CHANGE is to design system-level interventions to bring forward the date of effective use of NAMs. To achieve this goal, CHANGE follows a three-phase methodological approach “explore – reflect – design”:

Explore: Identify factors that might matter for the uptake and effective use of NAMs through assessing and describing mental models of the regulatory toxicology system by people operating within it.

Reflect: Prioritize the factors that matter by challenging the mental models and hypotheses created in the explore phase.

Design: Develop interventions addressing the factors that matter.

System diagrams visualize processes and dynamic forces in the regulatory toxicology system and the interactions between these forces will be used to identify opportunities for improving the effective use of NAMs. System diagrams with feedback loops will be used to identify interventions important for the effective use of NAMs. Reinforcing feedback loops in the system diagram shows where small changes will be amplified into big changes. Balancing feedback loops in the system diagram shows forces that will counteract changes. The system diagrams also enable identification of potential unintended consequences and indirect effects of interventions.

CHANGE includes an international group of people in the regulatory toxicology system having different roles and focuses, different specializations, working in different sectors with specific types of chemical substances covered by specific legislations. The project is based on involvement, networking, and dialogue.

Building on previous and ongoing efforts and initiatives, and focusing on system factors, CHANGE aims to be a catalyst for bringing forward the date of effective use of NAMs in the regulatory toxicology system.

Presentation: Oral

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3Rs integrating 3 worlds in Brazil: A case study

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Despite Brazil's long tradition of incorporating animal protection in its legislation, the successful implementation of the 3Rs has only been possible due to a joint effort by several stakeholders, including public and private sectors and non-profit organizations. The steps taken to replace the rabbit pyrogenicity test (RPT) are described here as an example. RPT is an *in vivo* method developed in the 1940s that uses mice to detect pyrogens (substances that produce a rise in body temperature) as part of the quality control of injectable products required by regulatory agencies worldwide. In 2019, following the recommendation of the Brazilian Center for the Validation of Alternative Methods (BraCVAM), the National Council for the Control of Animal Experimentation (CONCEA) recognized an alternative to RPT, the Monocyte Activation Test (MAT), a human-specific *in vitro* method that can detect both endotoxin and non-endotoxin pyrogens. The CONCEA Normative Resolution 45 recognizes the MAT and establishes the mandatory replacement of the RPT in 5 years. Technology transfer requires human resources training, which for the 3Rs in Brazil has been supported by the Ministry of Science and Technology through initiatives like the National Network of Alternative Methods (Renama), which organizes training to implement and perform recognized alternative methods. As non-animal data acceptance by regulators is a key factor for 3Rs implementation, since 2015 the Brazilian Health Regulatory Agency (Anvisa) has been committed to accepting data derived from alternative methods recognized by CONCEA. Another important aspect is the inclusion of non-animal methods in the Brazilian Pharmacopeia, exemplified by the recent inclusion of MAT, following the creation of a working group to write the chapter and a public consultation that took place in 2024. A key stakeholder for the transition to animal-free methods are the animal protection NGOs like the Humane World for Animals (formerly known as Humane Society International). By creating local working groups, sharing knowledge and experiences from foreign vaccine manufacturers and experts in animal-free methods, as well as organizing workshops and performing other activities to promote the implementation of non-animal methods in Brazil, a lot of effort has been put to identify the barriers and create strategies to overcome them. Specifically for the replacement of the RPT, we are currently working on promoting the adoption of a risk-assessment pyrogenicity strategy in Brazil, which includes a request followed by a collaboration with CONCEA for the recognition of the recombinant Factor C (rFC) method to replace the Limulus Amebocyte Lysate (LAL) test for the detection of bacterial endotoxins, and other activities including webinars to raise awareness and share experiences to ensure implementation and data acceptance. The replacement of the RPT is used here as an example of a multi-stakeholder strategy with several lessons learned that can be applied to replace other animal tests. As can be observed, the effective implementation of 3Rs in Brazil is the result of a joint effort by different stakeholders, each doing their part to ensure animal welfare and avoid the use of animals used for scientific purposes whenever possible. By sharing the Brazilian experience, we aim to encourage local stakeholders to continue their fruitful work and inspire other countries to achieve the same by promoting 3Rs for a better world.

Presentation: Oral



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The role of IATA in implementing NAMs in a regulatory context

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Next Generation Risk Assessment (NGRA) aims to refine, reduce and ultimately replace animal testing, and transition to mechanistically driven hazard and risk characterization. This is achieved using New Approach methodologies (NAMs), which include *in silico*, *in chemico* and *in vitro* approaches. In the context of food and feed safety, numerous jurisdictions exist, each with varying *in vivo* data requirements depending on the risk assessment context, such as regulated chemicals or contaminants. Currently, the European Commission, with the involvement of European Agencies (European Food Safety Authority (EFSA), European Chemicals Agency (ECHA), the European Medicine Agency (EMA)) as well as key stakeholders, is developing a roadmap for the phasing out of animal studies. This roadmap aims to provide short-term, mid-term, and long-term solutions. The Integrated Approaches to Testing and Assessment (IATA) developed by the OECD combines multiple streams of evidence, including historical *in vivo* and NAMs data, to conclude on chemical toxicity and fill data gaps through a weight of evidence (WoE) approach. Ideally, IATA supports the derivation of quantitative hazard and risk metrics for safe levels using toxicokinetic (TK) and toxicodynamic (TD) information, such as modes of action (MoA) and adverse outcome pathways (AOPs). This presentation aims to contextualize relevant problem formulations for RA of food and feed chemicals in relation to available data and tools, using tiered approaches integrated through a WoE approach in both data poor and data rich situations. Practical examples of using the IATA framework, integrating TK and TD information, will be illustrated for emerging contaminants, regulated chemicals, and historical contaminants with extensive hazard databases. Reporting WoE templates in a tiered fashion will also be discussed. Recent progress in *in silico* modelling including quantitative structure-activity relationship (QSAR) models, physiologically-based kinetic (PBK) models, and PBK-dynamic (PBKD) models for NGRA, will be highlighted as key enablers to further support the application of IATA in NGRA. The presentation will conclude with a discussion on critical data gaps and future research needs in the food and feed safety risk assessment areas.

Presentation: Oral

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Case study: Hazard characterization of agrochemicals/contaminants focused on DNT

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In the context of the European Union RISK-HUN3R project (with the ASPIS consortium), a novel and comprehensive next-generation risk assessment workflow, called ASPA, has been developed. Several case studies are ongoing to test and improve the ASPA framework, and its different modules. Three distinct DNT case studies have been developed and will be used to refine the respective parts of the ASPA workflow. Each case study targets specific ASPA modules and their interconnections. Risk assessment scenarios will be based on two groups of agrochemicals/food contaminants (strobilurin fungicides and neonicotinoids) to investigate how to deal with complex toxicokinetic queries. We explored how to account for intracellular concentrations, how to derive realistic points of departure (PoDs), and how to set thresholds for AOP activation by considering exposure time, developmental time, and dose concordance. The identification of uncertainties of all steps will provide information on safe margins of exposure. A high priority will also be given to defining the role of xenobiotic metabolism and how this module can be integrated into ASPA. Another important matter will be to define criteria on when and how to enter the (DNT) hazard module. One approach will be to define and benchmark assay alerts and hits. A third case study will investigate the potential use of ASPA for classification, packaging, and labeling (CLP) purposes. DNT toxicants would be classified as reproductive toxicants and a set of rules for such an approach will be defined.

Presentation: Oral



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PreMixT: An AI-enhanced platform for predictive modeling of chemical mixture toxicity

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Chemical risk assessment traditionally focuses on individual compounds, yet environmental exposures frequently involve complex mixtures whose combined toxicities remain poorly characterized. Accurate prediction of mixture toxicity demands robust computational tools incorporating rigorous data curation, innovative modeling approaches, and reliable validation methods. The present study introduces the PreMixT toolkit, an advanced artificial intelligence (AI)-based platform designed to predict mixture toxicity across multiple regulatory endpoints relevant to environmental and human health assessments. Here, we address regulatory calls for New Approach Methodologies (NAMs) aiming to minimize animal testing by delivering computationally efficient, accurate mixture toxicity predictions.

We collected comprehensive toxicity data from the Integrated Chemical Environment (ICE) database (<https://ice.ntp.niehs.nih.gov/>), curating acute systemic (oral, dermal, inhalation), skin and eye irritation, and skin sensitization endpoints following meticulous standardization procedures aligned with GHS and EPA guidelines. Chemical mixture data initially represented as separate components were combined into singular formulation-based records, carefully removing inconsistent entries, inorganic substances, salts, and conflicting replicates. QSAR models were developed using Random Forest algorithms with SiRMS descriptors, employing a stringent external 5-fold cross-validation protocol and internal Y-randomization tests to confirm model robustness.

For the acute oral toxicity dataset containing 440 mixtures, the QSAR model achieved high predictive accuracy (CCR = 0.80, sensitivity = 0.73, specificity = 0.87, PPV = 0.83, NPV = 0.79). The acute inhalation toxicity dataset (96 toxic vs. 84 non-toxic mixtures) yielded moderate predictive performance (CCR: 0.67, sensitivity: 0.66, specificity: 0.69, PPV: 0.71, NPV: 0.64). The acute dermal dataset exhibited severe imbalance (9 toxic vs. 540 non-toxic mixtures), resulting in a limited predictive utility. Models for eye irritation (CCR: 0.67, sensitivity: 0.87, specificity: 0.45), skin irritation, and skin sensitization faced similar challenges due to class imbalance. These results underscore the critical need for further dataset expansion and balanced data collection to enhance predictive model reliability across diverse toxicity endpoints.

The PreMixT platform represents a substantial advancement toward reducing reliance on traditional animal testing methods by providing regulatory agencies and industrial stakeholders with scientifically rigorous, reliable, and cost-effective predictive tools. Future developments will expand the PreMixT toolkit to incorporate additional toxicity endpoints, integrating novel mixture descriptors and advanced deep-learning methodologies to strengthen chemical mixture risk assessments.

Presentation: Oral

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Integration of human and environmental health education & training – Facilitated discussion

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After the presenters have described activities in several regions across the world, this facilitated discussion will explore workshop participants' feedback on any perceived silos between education & training initiatives for human health and the environment, identify ways to break these down and discuss how to successfully integrate human and environmental health in future educational & training initiatives.

Slido will be used to gain real-time audience interaction and engagement through live polls, Q&A, and interactive feedback.

Presentation: Oral

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Integrating new approach methods to inform chemical safety evaluation at the US EPA

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Chemical risk assessment is foundational to safely regulating chemicals and protecting human health. However, the traditional toxicology studies and risk assessment process is time and resource intensive and take 6-14+ years depending on the complexity of the studies and the assessment. As a result, less than 25% of the chemicals identified in the environment and less than 5% of chemicals in active commerce have human health assessments from US federal agencies. As the US Environmental Protection Agency (EPA) continues to implement operational efficiencies in its ability to assess the safety of chemicals for human health and the environment, the development and application of New Approach Methods (NAMs) has gained increasing traction. The EPA NAMs Work Plan, updated in 2021, articulates the Agency's roadmap and tangible steps toward pursuing and achieving a reduction in the use of vertebrate animals for chemical evaluations while remaining fully protective of human health and the environment.

Now in its final phases of completion, this talk will provide a retrospective assessment of the EPA NAMs Work Plan goals and key implementation milestones, that include: (1) evaluate regulatory flexibility for accommodating the use of NAMs, (2) develop baselines and metrics for assessing progress, (3) establish scientific confidence in NAMs and demonstrate application to regulatory decisions, (4) develop NAMs that fill critical information gaps, and (5) engage and communicate with stakeholders. This abstract does not represent official views or policy of the US EPA or of the US government.

Presentation: Oral



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Computational approaches to solve systemic toxicity questions and opportunities for thinking One Health in the pharmaceutical sector

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The requirements to assess the safety of medicines for humans have increased significantly over time, expanding beyond the active pharmaceutical ingredients (APIs) to include impurities arising from synthesis, formulation, manufacturing, packaging, and storing of drug products. This shift reflects scientific and technological advancements, as well as the growing demand for more holistic, risk-based safety evaluations. As regulatory requirements become more stringent, an increasing number of chemicals must be assessed, many of which are data-poor and difficult to test experimentally. This makes computational toxicology an essential tool in the risk assessment of these substances. Computational approaches such as (Q)SAR ((Quantitative) Structure-Activity Relationship) models, PBPK (Physiologically Based Pharmacokinetic) models, and read across can facilitate early hazard identification, support data gap filling, and contribute to reduce reliance on animal testing at different stages of drug development, both for new APIs and generic drugs. Beyond human health considerations, the relevance of environmental safety assessment is becoming increasingly recognized in pharmaceutical risk management. Although environmental risk assessments are mandated only in certain regions, the evolving regulatory landscape aligns with the One Health concept, which recognizes the interconnectedness of human, animal, and environmental health. As safety evaluations expand to incorporate environmental considerations, the pharmaceutical sector faces both opportunities and challenges in integrating holistic, cross-disciplinary approaches into safety assessment and regulatory decision-making. The transition to a One Health-centered paradigm necessitates further advancements in data integration, computational modeling, and in regulatory harmonization. This presentation discusses the regulatory uptake of computational approaches for systemic toxicity in the pharmaceutical sector and explores the opportunities for further integration of these methods within the evolving regulatory landscape regarding the safety of pharmaceuticals and the One Health concept.

Presentation: Oral

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Leveraging non-animal methods for acute toxicity in the Brazilian regulatory context

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Acute oral toxicity testing is a critical component of regulatory safety assessments across various sectors in Brazil, including pharmaceuticals, cosmetics, agrochemicals, medical devices, toys, schooling materials, and for the classification and labeling of chemicals under the Globally Harmonized System (GHS), which is internalized through the Brazilian Association of Technical Standards (ABNT) document ABNT NBR 14725:2023. As in other regions of the world, compliance with these regulatory requirements has traditionally relied heavily on animal testing. The adoption of alternative methods for acute oral toxicity has been driven by global efforts to reduce animal testing, leading to the development of OECD test guidelines with non-animal and refined approaches. In Brazil, this progress was formally recognized with the publication of Normative Resolution RN 18/2014 by the National Council for the Control of Animal Experimentation (CONCEA). This resolution recognized three reduction-based methods to replace the traditional LD50 test: the Fixed Dose Procedure, the Acute Toxic Class Method, and the Up-and-Down Procedure, all of which significantly reduce the number of animals required while still providing hazard identification data. Additionally, OECD Guidance Document 129, also recognized on RN 18/2014, describes an *in vitro* cytotoxicity assay that can estimate the oral LD50 value, supporting a weight-of-evidence approach and further contributing to the reduction of animal use in acute oral toxicity testing. Despite these regulatory advancements, the adoption of non-animal approaches for acute oral toxicity remains limited, both in Brazil and globally. However, ongoing progress in *in vitro* models, *in silico* approaches, and integrated testing strategies continue to offer promising alternatives to animal testing. Demonstrating the fit-for-purpose applicability of these methods and establishing an adequate level of confidence in their predictive performance will be crucial for their regulatory acceptance, paving the way for a modernized, human-relevant assessment of acute oral toxicity. As Brazil advances its regulatory framework for industrial chemicals, including the recent enactment of Law 15022/2024, the demand for toxicity data is expected to grow. Keeping pace with international advancements in non-animal methods will be essential for ensuring robust, efficient, and ethical safety assessments. Continued investment in method validation, regulatory acceptance, and innovation will not only support compliance with evolving regulations but also strengthens Brazil's ability to address current and future safety assessment challenges.

Presentation: Oral



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Machine learning in toxicology: Best practices for supervised and unsupervised approaches

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In the field of machine learning, two primary methodologies – supervised and unsupervised learning – serve distinct purposes and are employed based on data characteristics and desired outcomes. Supervised learning involves training algorithms on labeled datasets, allowing models to generate accurate classifications or predictions based on input data. Common techniques include decision trees, support vector machines, and neural networks, which are effective when historical data informs future forecasts.

In contrast, unsupervised learning seeks to identify hidden patterns within unlabeled data. By utilizing clustering and dimensionality reduction techniques, such as k-means clustering and principal component analysis (PCA), algorithms reveal inherent data structures, grouping similar samples without predefined classifications. This approach is particularly beneficial for exploratory data analysis, helping to uncover insights and relationships that might not be immediately apparent.

In this talk, we will discuss best practices for choosing and applying both supervised and unsupervised learning techniques, emphasizing the importance of aligning the chosen method with research objectives and context. We will also highlight the concept of similarity, illustrating its relevance in practical exercises.

Several case studies will demonstrate the synergy between these methodologies. One such example is the Pred-Skin sensitization web tool, which integrates multiple Quantitative Structure-Activity Relationship (QSAR) models based on diverse data sources, including *in vitro*, *in chemico*, and both mice and human *in vivo* studies. This tool employs a consensus naïve Bayes model to predict human sensitization effects from chemical structures, showcasing how supervised learning can complement unsupervised methods to improve predictive accuracy in toxicology.

Another case study is the Cyto-Safe platform, which utilizes a curated dataset of approximately 90,000 compounds tested on 3T3 mouse fibroblast and HEK 293 human embryonic kidney cell lines. It employs Extended Connectivity Fingerprints (ECFP4) and Light Gradient Boosting Machine (LightGBM) algorithms to classify compounds as cytotoxic or non-cytotoxic.

The Pred-hERG 5.0 tool was developed using QSAR models that included additional datasets and updated classifiers, introducing regression models for predicting hERG activity (pIC50). Using data from ChEMBL v30, the new binary and multiclassification models surpassed previous iterations of Pred-hERG and existing public models. Notably, the regression model achieved an R^2 of 0.61 and an RMSE of 0.48, outperforming all previously published regression models.

By examining these best practices and case studies, we aim to provide researchers and practitioners with valuable insights into effectively leveraging machine learning in their toxicological assessments. Understanding when to utilize supervised versus unsupervised learning is crucial for advancing research in toxicology and other data-driven fields. Ultimately, mastering these techniques is essential for making informed decisions and driving innovations in research applications.

Presentation: Oral

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Fit-for-purpose computational toxicology tools: Considerations for regulatory use

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With the continued refinement and application of new approach methodologies (NAMs), opportunities to leverage mechanistic insight and computational modeling as lines of evidence in a decision-making context are becoming more feasible. More specifically, adapting NAMs designed for research and early-stage screening/prioritization into data with utility for late-stage mechanistic decision-making requires thoughtful review to ensure fit-for-purpose application. This shift in how NAMs and their resulting data are used requires careful consideration regarding the scrutiny for acceptable performance criteria and reporting that must be considered to substantiate interpretations and conclusions in a new context. This talk will provide examples of the challenges and opportunities for refining data reporting and fit-for-purpose application of *in silico* tools to build confidence in leveraging these resources in a decision-making context, including comparing/contrasting what outputs may be acceptable for research vs. regulatory applications. For example, identifying key information required for replicating computational workflows, with an emphasis on versioning and source data/code transparency, and establishing reporting templates that help guide consistent documentation of meta-information, metrics, and data summaries will be critical to the successful implementation of *in silico* tools. From PBK, QSAR, and other mathematical or statistical approaches to artificial intelligence and predictive modeling, attendees will gain high-level insight into how *in silico* NAMs are developed, utilized, and optimized for specific contexts of use to confidently assure appropriate use and interpretation of results.

Presentation: Oral



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Towards regulatory transformation to enable animal-free safety assessment of chemicals and consumer products

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Today, a growing number of regulatory frameworks stipulate that animal testing must only be conducted as a last resort. Whilst such policy measures are indeed vital, the integration of nonanimal methods (NAMs) to regulatory practice and guidance remains inconsistent and poorly enforced, leading to a climate in which the “last resort” has not been upheld (Macmillan et al., 2024). In the worst case, routine animal testing persists even for endpoints for which validated and internationally recognized NAMs have been available for decades.

A certain amount of the reason for this can be attributed to rigid legislative frameworks that have been built on the presumption of animal models being the gold standard of assessing safety; however, regulatory practice and interpretation also plays a role in the conduct of animal testing where NAMs are available.

Humane World for Animals is working with partners from industry, including NAM developers and consulting firms as part of the Animal-Free Safety Assessment Collaboration (AFSA) to drive regulatory transformation. Through focused stakeholder outreach in eight strategic global markets, we will establish regulatory precedent by providing specific guidance to drive the use of NAMs and ensure last resort requirements can be easily identified and enforced. This guidance is based on in-depth scientific analysis of the tools and techniques that are currently available and ready for regulatory use. Once this precedent has been set, we can further integrate next-generation risk assessment (NGRA) and animal-free safety assessment concepts to drive global consistency in phasing-out the use of animals for regulatory assessments.

In many cases, regulatory transformation can only be achieved by increasing confidence and understanding in leveraging NGRA tools and concepts. The AFSA Master Class is a unique, open-source, Master’s-level course that will enable individuals and organizations to upskill and build capability in this space, opening the door to more rapid and effective regulatory change.

Through leveraging science-led regulatory engagement, effective stakeholder dialogue, and building capability, Humane World for Animals and AFSA are driving fundamental and lasting change in global chemicals regulatory assessment, and building a foundation for integrating NGRA and animal-free science into regulatory structures.

Presentation: Oral

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An advanced microphysiological system for genotoxicity assessment

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The assessment of genotoxicity for test compounds relies on a combination of tests designed to evaluate various endpoints associated with human diseases: mutagenicity, clastogenicity and aneuploidy. Currently, no single test can detect all mechanisms effectively, as existing assays involve rodent metabolic activation systems or *in vivo* rodent testing. This reliance can lead to discrepancies in human accuracy and predictive responses.

This study aimed to develop a single *in vitro* assay capable of accurately addressing both *in vitro* and *in vivo* genotoxicity endpoints using a human cells metabolically competent *in vitro* system to improve human-relevant predictivity and replace the reliance of animal testing. Different models were evaluated, and the most promising evaluated model consists of a fluidic flow microphysiological liver-on-chip system. This system, supported by CNBio’s PhysioMimix™ platform, incorporated HepaRG cell line on specialized Liver Plates (MPS-LC12), which enhanced liver cells health and functionality compared to previous tested performed on Barrier Plate (MPS-T12).

Preliminary results using direct and pro-genotoxicants requiring metabolic activation demonstrated outcomes consistent with *in vivo* response. These included results from the comet assay, and micronucleus test. Key measurements, such as urea production and albumin secretion confirmed appropriate liver functionality and metabolic competence.

In conclusion, the human cells system using HepaRG cells in Liver Plates from CNBio represents a promising model, demonstrating appropriate metabolic properties without requiring additional metabolic activators. This system effectively addresses genotoxic adverse outcomes within a single system, i.e., induction of chromosomal damage or damage to the mitotic apparatus (micronucleus test) and DNA strand breakage (comet assay).

Presentation: Oral



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The trust-building capacity of validation

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Validation was developed as a process to assess the scientific foundation, reproducibility, and relevance of toxicological test methods, establishing itself as a crucial step for regulatory adoption. While applicable to both *in vivo* and experimental new approach methods (NAMs), the validation process was primarily shaped by *in vitro* test methods designed to replace traditional animal testing for acute human health effects on a one-to-one basis. Through a collaborative effort involving all stakeholders and guided by the principles of independence and transparency, the process was formalized, resulting in a comprehensive set of rigorous requirements to ensure reliable validation outcomes.

After more than two decades, adapting the validation framework to reflect scientific progress – while addressing fundamental challenges – has become imperative. Regarding technical and scientific hurdles, the strategic integration of multiple NAMs to assess complex toxicological effects raises a key question: how should these methods be validated individually? In particular, relevance assessment needs to be focused on the specific mechanistic step being addressed while ensuring that the validation provides the necessary information for integration. Another significant challenge is the limited availability – or complete absence – of reference test method results for comparison, which can severely impact both test substance selection and relevance assessment. Additionally, concerns have been raised regarding the efficiency of validation, particularly its rigidity and resource intensity. However, the current debate does not fully leverage two decades of validation experience, which could be used to transparently consolidate existing data and provide an evidence-based foundation for discussions.

In summary, modernizing validation while addressing these challenges is essential, and several initiatives are already underway. However, any proposed solutions must preserve the trust-building function of validation. Validated NAMs must maintain a high level of credibility to be considered valuable for regulatory decision-making and must support decisions that ensure adequate protection of human and environmental health. Undermining either aspect could significantly erode regulatory and public trust in NAMs, ultimately delaying their effective implementation and the long-awaited transition away from animal testing.

Presentation: Oral

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Classification for mutagenicity based on non-animal methods only: A dream or reality?

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The UN Globally Harmonized System for Classification and Labelling (GHS) has established criteria for the classification of chemicals to facilitate chemical risk management and transport. Currently, mutagenic substances are classified within the hazard class germ cell mutagenicity, through classification criteria, which rely mainly on *in vivo* data. While proven mutagens in germ cells are classified in category 1, somatic cell mutagens are classified in category 2. The GHS criteria are currently being revised to clarify issues that have brought to difficulties in their interpretation, consider current state of science and facilitate hazard classification using non-animal methods. To support this revision, a study was conducted to determine whether there are situations where the standard *in vitro* tests alone are sufficient to classify substances as mutagenic without the need for *in vivo* confirmation. The study analyzed over 700 chemicals from the EURL-ECVAM genotoxicity and carcinogenicity database exploring various combinations of *in vitro* tests that cover different genotoxicity endpoints, such as gene mutation, clastogenicity and aneugenicity. When the *in vitro* results were compared with the overall *in vivo* assay outcome, they showed that most combinations of *in vitro* tests that were positive for all endpoints were confirmed *in vivo*, with at least one positive study. Interestingly, a closer examination of the outliers revealed that most of them could be explained by underlying factors, providing a clear understanding of why they yielded misleading positive results. These findings suggest that, in some cases, it may be possible to classify a chemical as a somatic cell mutagen using *in vitro* assays alone.

The presentation will also explore additional considerations discussed in the context of the GHS revision that aim at reducing reliance on animal tests. Specifically, it will cover the potential of novel *in vitro* assays that address specific biological mechanisms providing supplementary data, the role of non-testing methods and the question whether a carcinogenicity study is necessary when a substance is already known to be mutagenic.

Presentation: Oral



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Human 3D spheroids for genotoxicity assessment

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Genotoxicity testing has traditionally relied on two-dimensional (2D) *in vitro* cell models, which are limited in their ability to replicate the complex metabolic interactions observed *in vivo* and are restricted to an acute 24-hour treatment. In particular, 2D models have a poor metabolic property, an important step for some key genotoxic carcinogens. In recent years, three-dimensional (3-D) *in vitro* cell models have emerged as potentially more physiologically relevant systems. However, they have not allowed for high throughput genotoxicity screening and have not been standardized. Within the framework of the European Partnership for the Assessment of Risks from Chemicals (PARC; GA: 101057014), we developed a human multicellular liver spheroid containing immune, hepatic and Kupffer stellate cells. We combined the HepaRG cells (hepatocytes and cholangiocytes), the hTERT-HSC cell line (Kupffer cells) and the THP-1 cells (immune cells) to create metabolically competent human liver spheroids. We were able to grow spheroids, perform acute or repeated chemical treatments, indirect antibody immunofluorescence staining and confocal imaging on the same plate with four spheroids per well. An image analysis workflow was then developed using 3D reconstructed images. First, we demonstrated the important metabolic capacity of this 3D model using numerous cytochrome P450 substrates. Chemical genotoxicity evaluation with several biomarkers, in particular related to specific genotoxicity modes of action (γ H2AX and pH3) will be presented and the difference between the 2D and 3D cell models will be highlighted. The difference between acute and repeated treatment will also be detailed. Other specific biomarkers related to inflammation and oxidative stress have also been implemented in this model. Using these spheroids, we were able to detect genotoxic potential for some chemical not efficiently detected in 2D models. This new human multicellular liver spheroid model may allow more efficient identification of chemicals with genotoxic and carcinogenic potential in the future.

Presentation: Oral

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Explainable artificial intelligence models for developmental and reproductive toxicity prediction using ToxCast data in cross-species adverse outcome pathway

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Artificial intelligence (AI) models offer new opportunities to evaluate the potential toxicity of numerous environmental chemicals. However, many predictive toxicity models remain inherently black boxes problem, presenting challenges for toxicologists in interpretation and hindering their regulatory acceptance. The complexity of mechanisms underlying apical toxicities, combined with a lack of mechanistic evidence, can raise doubts about model reliability, potentially leading to coincidental or inaccurate results. The adverse outcome pathway (AOP) framework holds promise in addressing this issue. AOPs can link molecular toxicity endpoints to human and ecotoxicity endpoints. In response, this study aimed to develop explainable artificial intelligence models for predicting toxicity using ToxCast data within an AOP framework. In this study, we developed explainable AI models within an AOP framework to predict chemical toxicity using ToxCast data. First, we identified AOPs associated with reproductive toxicity from the AOP Wiki. We then collected *in vitro* bioactivity data from ToxCast and *in vivo* toxicity data from ECOTOX DB. These data were integrated into AOPs, and each assay-AOP pair underwent further assessment to determine relevance to taxa within ecosystem for cross-species. Finally, machine learning models were developed to predict each mechanistic and apical endpoint-based toxicity. As a result, models for each key event and apical endpoint achieved high performance (F1 score: 87-92%). In a proof-of-concept case study, the model improved the sensitivity of predictions compared to models developed based on *in vivo* toxicity data alone. This study can serve as a guide for developing explainable machine learning models based on AOPs, addressing the current primary bottleneck in using AI models for chemical risk assessment.

Presentation: Oral



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North America perspective

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There are many benefits to a holistic approach to assessing potential chemical risks to human health and the environment, especially with New Approach Methods/Non-Animal Methods (NAMs). Information from pathway-based approaches, cheminformatics databases, and upstream mechanistic assays can provide information across a variety of species, and the information gained from high-throughput testing initiatives can provide a veritable feast of data with which to understand chemical impacts on multiple species, provided researchers, companies, and regulators have the knowledge and frameworks to interpret such data. In North America, the US Environmental Protection Agency has been working to develop assessment methods, as well as frameworks and case studies to facilitate interpretation to address specific needs by federal, regional, and state authorities. Educational activities are essential to the uptake and use of these approaches. They can be challenging to undertake given the complexity of the approaches, the diverse backgrounds of data users, and the need to engage advocacy organizations and the public to facilitate the embrace of decision-making based on new approaches.

Presentation: Oral

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Success story in approval of *in vitro* potency tests for inactivated rabies vaccines

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Historically inactivated Rabies vaccines for human and veterinary use have been potency released using the NIH mouse challenge test and more recently with a refined serology alternative in the EU. Work towards the substitution of this test with an *in vitro* immunological test for the key glycoprotein immunogen has been on-going for many years and in the case of Veterinary vaccines is complicated by the presence of different adjuvants in the vaccines. We report here the success story from 3 different global manufacturers in developing, validating and gaining regulatory approval for 3 different types of ELISA based assay for Rabies Glycoprotein. Although the tests methods vary, we will highlight the common approaches to the substitution. Demonstrating method and reagent suitability and discriminatory power as well as VICH validation following the principle of EP monograph 5.2.14. and addressing specification setting at release and through shelf-life using a consistency approach.

Presentation: Oral



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Challenges to the credibility and human relevance of using *in vivo* data for validation purposes

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Regulatory authorities have traditionally used *in vivo* study results to make safety decisions. These studies formed the basis for classification and labeling systems used by national and international authorities with the goal of protecting human and environmental health. Accordingly, the results from these studies have been used to characterize the usefulness and limitations of an alternative approach, following a very structured process for conducting and evaluating inter laboratory validation studies to do so. This process focused on a single method that was being proposed as an alternative to an existing method; a one-to-one replacement whereby the proposed replacement would be just as health protective as (if not more than) the reference (*in vivo*) test method. However, a variety of factors including inter-animal and inter-laboratory differences create variability in animal studies, confounding direct comparisons with *in vitro* results. This raises questions about the “gold standard” for validation, as *in vivo* data itself has limitations and is not a perfect benchmark. The questionable human relevance of *in vivo* models for certain endpoints further complicates the validation process. For example, differences in the anatomy of the rabbit and human eyes, and differences in modelling exposures in rabbit eyes relative to human eyes. Given this multitude of challenges associated with available reference data, why would we expect a New Approach Methodology to always reproduce the *in vivo* outcome? Therefore, we seek new approaches to demonstrate the usefulness and limitations of non-animal methods to establish confidence in their regulatory application for identifying all ranges of toxicity and associated hazards. This presentation will highlight variability analyses for several endpoints, discuss human relevance (or lack thereof) and the importance of their consideration in evaluating the performance of NAMs.

Presentation: Oral

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Alternative methods in Brazil: From NAMs development to data acceptance

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Advances in this field have been accompanied by key regulatory milestones, such as the Toxicology for the 21st Century report in 2007; the enactment of the Arouca Law and the establishment of the National Council for Animal Experimentation Control (CONCEA) in Brazil in 2008, which regulates the use of animals in research and education; the European Union in 2013 ban on animal testing for cosmetics; the U.S. FDA Modernization Act of 2023, which removed the requirement for animal testing; and Brazil’s 2023 law mandating the adoption of alternative methods for cosmetics evaluation. Most recently, in November 2024, Brazil enacted Law 15,022, which establishes the National Inventory of Chemical Substances and sets regulations for the risk assessment and control of chemicals produced or imported into the country. This presentation will highlight the example of the Laboratory of Education and Research in *In vitro* Toxicology at Federal University of Goiás, Brazil, in implementation and development of Alternative Methods and NAM in Brazil. We have focused on cross-sectoral collaboration with academia, industry, CROs and regulators to use non-animal-based chemical assessments throughout Brazil and Latin America implementing and developing Alternative Methods and NAMs, as well as, training researchers and students, assisting researchers and communicating the advanced new methodologies to accelerate adoption and regulatory acceptance.

Presentation: Oral



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Overview about recent European 3R activities with a focus on 3R centers and the COST Action IMPROVE

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This presentation will give an overview of the current developments in the field of 3Rs in Europe. The development and growth of 3R centers in Europe is tightly linked to political activities. One of the lighthouse projects is the COST Action IMPROVE.

IMPROVE was developed from a bottom-up approach initiated by members of European 3R centers and its network platform EU3Rnet. COST Actions are European projects that promote the formation of networks.

This activity will be presented in detail with its possibilities, plans and toolbox. The working groups focus on Quality and Translatability of Science, Implementation, Dissemination and Education. Ethics are an integral part in the work of the individual working groups. Currently, about 250 participants, reflecting several stakeholder groups, take part in the activities of this project. Cooperation and active involvement in the COST Action is encouraged within the framework of this open, bottom-up network approach, including also international partners. Recent activities will be presented, such as surveys of animal caretakers, a NAMs expert database, training schools, 3Rs workshops, reports and publications. An additional focus will be the collaborations of the network of European 3R centers (EU3Rnet). Further information can be found via the following link: <https://cost-improve.eu/>

Presentation: Oral

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InsightAI: An intelligent application for risk assessment of chemicals

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Computational models powered by artificial intelligence (AI) have emerged as reliable, rapid, and cost-effective tools for drug design and toxicity evaluation in the early stages of drug discovery and chemical safety assessments. This presentation will introduce LabMol InsightAI, an intelligent application developed by the LabMol research group aimed at accelerating drug discovery and enhancing the risk assessment of chemicals. LabMol InsightAI leverages the latest advancements in AI, machine learning (ML), and cheminformatics to provide researchers with a comprehensive and efficient platform for drug discovery and toxicity research. Its suite of features streamlines workflows, improving research efficiency, particularly in risk assessment initiatives. Key functionalities include: Automated Dataset Preparation and Curation: Facilitating the organization and refinement of chemical data for analysis. QSAR Modeling: InsightAI enables researchers to construct predictive quantitative structure-activity relationship (QSAR) models. These models classify compounds as active or inactive and predict their biological activity or toxicity based on chemical structures. This capability is crucial for virtual screening across large chemical databases, allowing for the identification of potential drug candidates and toxicants. Machine Learning-Based Toxicity Prediction: Utilizing state-of-the-art machine learning algorithms, InsightAI predicts the toxicity of chemicals from their structures, significantly diminishing reliance on expensive and time-consuming animal and *in vitro* testing. Mechanistic Interpretation: InsightAI offers valuable insights into the mechanisms underlying chemical-biological interactions, enhancing the understanding of drug and toxicant modes of action and potentially revealing new targets for drug development. User-Friendly Interface: The intuitive design of InsightAI allows researchers to visualize and analyze data easily, build models, and interpret results efficiently.

One case study highlighted will focus on the Pred-EDC project, which employs QSAR-AI models to predict endocrine-disrupting chemicals (EDCs) affecting the progesterone receptor (PR). The developed AI models demonstrated impressive performance metrics: for agonist compounds, the models achieved a balanced accuracy (BACC) of 0.82, sensitivity of 0.79, specificity of 0.83, and a Matthews correlation coefficient (MCC) of 0.56. For antagonist compounds, the models recorded a BACC of 0.81, sensitivity of 0.80, specificity of 0.82, and an MCC of 0.57. These results underscore the robustness of the models in effectively distinguishing between active and inactive compounds concerning PR. In addition to enhancing EDC prediction, this project aligns with the principles of the “3Rs” (reduction, refinement, and replacement) in animal experimentation. It highlights the potential of AI to minimize reliance on animal testing by offering reliable *in silico* alternatives. The integration of AI technologies with chemical data provides powerful insights for better understanding and managing the risks associated with EDC exposure, ultimately benefiting both public health and environmental safety.

Presentation: Oral



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Alternative strategies in surgical training

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The use of laboratory animals has been the gold standard for training in disciplines such as surgery, being a controversial issue that is not limited to the scientific field, having legal implications, conscientious objection and impact on the media and public opinion. The objective is to demonstrate how training and education methods have been modified over the years in a Public Pediatric Hospital to achieve acceptable levels of learning.

Through alternatives such as: use of inanimate material, simulation, software, reuse of cadavers, tissue preservation and 3D printing, a permanent training program was developed.

By comparing learning results generated by the alternative methods, additional benefits were obtained such as time and cost savings, improved potential for parameterization and repetition of the learning exercise, increased surgeon confidence and satisfaction, greater compliance with legislation on the use of animals, elimination of objections to the use of animals sacrificed for specific purposes and the integration of clinical perspective and ethics in the early stages of training of surgeons.

Evidence shows that educators can better serve their students, while minimizing financial and time burdens, with the introduction of well-designed training methods that do not depend on the harmful use of animals.

The gradual implementation of low-cost and high-tech models make a perfect match in the tools necessary to carry out such training Training and Capacity Building Task.

The design of the prints begins with the DICOM (Digital Imaging and COmmunications in Medicine) images of tomography or magnetic resonance from which the segmentation and subsequent printing of the region to be treated is performed. From a cost-benefit perspective, 3D printing-assisted surgery is an attractive alternative for those institutions that cannot afford navigated procedures. From these digital models, it is also possible to develop patient-specific instruments, such as cutting guides, assemblies or guides for implant placement.

The experience of both the surgeon and the bioengineers is limited and requires constant feedback between the surgeon's surgical needs and the alternatives that the bioengineer can offer.

The use of 3D reconstruction technology of the anatomy of congenital heart disease proved to be a tool that allows the creation of surgical plans based on more detailed knowledge of the different defects of each patient.

The work of an interdisciplinary group of different professionals who can analyze the same information through different methods is very important.

We are therefore at a turning point. Science and Society are advancing compared to a few years ago, but there are still many points to cover and many obstacles to overcome. It is therefore important that the scientific community continue to make efforts to support alternative methods, to continue investing in their development and implementation in all areas, since in this way we will all benefit: humans, because we will develop more reliable models, and non-human animals, because we will avoid unnecessary

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STopTox and PreS/MD: *In silico* NAMs for systemic and topical toxicity

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Modern chemical toxicology is facing a growing need to Reduce, Refine, and Replace animal tests for hazard identification. The most common type of animal assays for acute toxicity assessment of chemicals used as pesticides, pharmaceuticals, or in cosmetic products is known as a "6-pack" battery of tests, including three topical (skin sensitization, skin irritation and corrosion, and eye irritation and corrosion) and three systemic (acute oral toxicity, acute inhalation toxicity, and acute dermal toxicity) end points. We developed STopTox, a comprehensive collection of computational models that can be used as an alternative to *in vivo* 6-pack tests for predicting the toxicity hazard of small organic molecules. Whilst STopTox has been mostly developed for cosmetics, drugs and pesticides, we also developed PreS/MD for assessing the skin sensitization for medical devices. To develop these tools, we compiled, curated, and integrated the largest publicly available datasets and developed an ensemble of QSAR models for all existing regulatory accepted (or nominated as NAMs) assays. All the models were validated according to the best practices in the field OECD QSAR principles, using data on compounds not included in the training sets. Independent external evaluation performed in collaboration with NIEHS and Inotiv also confirmed high predictive power of the developed models. We established a publicly accessible web portals for STopTox (<https://stoptox.mml.unc.edu/>) and PreS/MD (<https://presmd.mml.unc.edu/>) integrating all developed models. Scientists and regulators can use these portals to identify putative toxicants or non-toxicants in chemical libraries of interest.

Presentation: Oral



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Lingering consequences of the great fish pain debate

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The complex life of fish has been underestimated for a long time, with fish welfare often being reduced to mere production endpoints and what seems a chronic debate over whether fish experience pain [1]. Consequences of this disconnect show across all sectors in which humans use fish, including their use in laboratories for scientific purposes. 1,218,766 fish were used for scientific purposes in 2022 in the European Union, which represents 17% of all animals that were used [2]. These high numbers can be associated with controversial ethics, including the question whether replacing certain mammals with fish in experiments – because their sentence is often considered to be less developed and their breeding, housing and testing is possible at lower costs – can truly be accepted as refinement according to the 3 R approach.

This presentation aims to remind us of our collective responsibility for all animals, regardless of how much we know about them and what level of capacity to experience pain we assume in them. It provides a) a comprehensive overview of the most recent statistics and developments on use of fish for scientific purposes in the EU, b) insights from recent studies that elucidate the intricate traits, skills and needs of fish, with focus on species groups prominently used in experiments, and c) raises concern over certain attitudes, procedures and regulations concerning fish in the context of animal experimentation that indicate insufficient protection of these animals. Concluding remarks will also transfer this discussion to other aquatic animals who are being used for scientific purposes.

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The role of computational biokinetic models in enabling DART-NAMs hazard characterization

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For the application of the New Approach Methods (NAMs) within the Next Generation Risk Assessment (NGRA), a major challenge remains the contextualization of *in vitro* toxicodynamic data to the respective equivalent *in vivo* effect doses. This process, known as Quantitative *In vitro* to *In vivo* Extrapolation (QIVIVE), relies on reverse dosimetry for the calculation of an external dose for risk assessment, assuming that equal unbound concentrations at the target site *in vitro* and *in vivo* correspond to equal adversity. As exposure situations differ fundamentally between the *in vitro* and the *in vivo* systems, such extrapolations require the integration of kinetics.

Physiologically based kinetic (PBK) models simulate *in vivo* absorption, distribution, metabolism, and excretion (ADME) using organism physiology and chemical properties. At the same time, *in silico* mass balance models can be used to predict *in vitro* compound distribution, considering system characteristics and physicochemical properties. Together, these biokinetic models bridge *in vitro* unbound concentrations to *in vivo* plasma or tissue levels, enabling external dose estimation for QIVIVE. Driven by the need to eliminate animal data, NGRA necessitates the optimized development and validation of kinetic models from *in vitro*, *in silico*, and historical data. Aligning with the parsimony principle, the models shall be as complex as necessary, ranging from simple high-throughput PBK models to models with more complex and mechanistic structures.

Traditional developmental and reproductive toxicity (DART) assessments often require a large number of animals and are, therefore, a priority for a rapid transition to NAMs. In the current presentation, the role of *in vitro* and *in vivo* gestational kinetic models in the interpretation of DART-NAMs will be described, with a focus on pregnancy. Here, PBK models must account for gestational physiological alterations, placental barrier function, and fetal development changes. Available maternal-fetal modelling platforms, as well as methodologies and gaps for the parameterization of chemical placental transfer will be discussed. QIVIVE DART case studies focused on developmental neurotoxicity (DNT) studies will be used as examples to illustrate the differences between approaches in a tiered risk assessment and to discuss their advantages and limitations.

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Gene-environmental interactions in autism, modeling maternal inflammation *in vitro*

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Autism spectrum disorder (ASD) arises from complex gene-environment interactions, yet traditional animal models often fail to capture these dynamics. Rodent maternal immune activation models provide insight into neurodevelopmental impacts of inflammation, but they cannot fully recapitulate human-specific genetic factors (like the 16p11.2 deletion) or the intricacy of human brain development. Such limitations underscore the need for human-relevant models to study how genetic susceptibility and maternal inflammation interact in ASD.

Here, we established a human *in vitro* model: cortical brain organoid assembloids (incorporating microglia) derived from induced pluripotent stem cells carrying a 16p11.2 deletion (with neurotypical controls for comparison). These organoids were challenged with a maternal inflammation-mimicking stimulus to model an *in utero* immune environment. In parallel, we isolated extracellular vesicles (EVs) from the organoid cultures and performed transcriptomic analysis to identify brain-specific EV RNA cargo. EV transcripts from organoids were compared to their cellular transcriptome and to EV profiles in human plasma to confirm neural specificity.

The 16p11.2 deletion organoids exhibited a blunted baseline cytokine profile but mounted an exaggerated inflammatory response under immune challenge, secreting more pro-inflammatory cytokines than controls. This hyper-responsiveness was accompanied by heightened microglial activation (evidenced by elevated CD68 and TREM2), indicating that the 16p11.2 mutation amplifies vulnerability to maternal inflammation. Notably, organoids derived from individuals with clinical ASD showed stronger responses than those from non-ASD donors despite the same deletion, highlighting gene-environment interactions as a driver of phenotypic diversity. Meanwhile, EV profiling revealed hundreds of brain-enriched mRNAs (predominantly related to synaptic function and myelination) within organoid-derived EVs. These transcripts overlapped with brain-specific mRNAs identified in circulating plasma EVs, underscoring the neural origin of the EVs. The EV cargo only partially mirrored the organoids' intracellular transcriptome, suggesting selective packaging and release of specific RNAs by neural cells. Collectively, these findings validate neural EVs as potential blood-accessible biomarkers of brain status.

In summary, this human stem cell-derived brain model recapitulates key ASD-related gene-environment interactions that are challenging to model in animals – exemplified by the interplay between a major genetic risk (16p11.2 deletion) and maternal immune stress. We further demonstrate that neural EVs carry brain-specific molecular signatures, which could be leveraged as non-invasive “liquid biopsies” to monitor neurodevelopmental perturbations (e.g., assessing maternal inflammation's impact on the fetal brain) in humans. By replacing animal models with human-relevant organoid systems and EV analysis, our approach offers a more translational and ethical platform for studying autism, aligning with 3Rs principles and advancing alternatives to animal use.

Presentation: Oral

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EPAA designathon systemic toxicity challenge

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New Approach Methods (NAMs) are increasingly used within industry to make decisions about the human safety of chemical exposures prior to manufacturing new products. This project aims to provide a cross Industry/EC environment for creative appraisal of current use of NAMs / non-animal science for decision-making.

Following a “deep-dive” workshop in 2021 and two EPAA Partners Forums on “Exposure Considerations for Human Safety assessments” in 2022, several areas where progress can be made to increase NAMs uptake in regulatory decisions making were identified: (i) examine how exposure-based approaches could fit into REACH, (ii) survey existing weight of evidence (WoE) approaches and evaluate potential use to characterize chemical hazards (case studies), and (iii) to investigate a tiered approach as an alternative Classification and Labelling (C&L) system for risk management, without animal data. The group agreed to focus on exploring the use of NAMs for classification of chemical-induced systemic human health effects.

To facilitate this, EPAA launched the pilot phase of this work, the “NAM Designathon 2023” Challenge for human systemic toxicity, which seeks to develop a chemical classification system capable of categorizing chemicals based on their bioactivity and their potential systemic availability using non-animal data. It was conceived as a collaborative approach which would involve any scientists interested in co-creating a new NAM-based approach to hazard classification. The solution does not need to predict outcomes of the animal studies/reproduce existing classifications but should reflect three levels of concern (high, medium, and low) associated with chemicals within the current GHS classification addressing systemic toxicity. EPAA provided a list of 150 chemicals and a reporting template.

Twenty-three teams submitted prototype solutions to the Designathon challenge. These were discussed in a dedicated workshop, with poster sessions and breakout groups in March 2024. Participants explored the technical aspects of the NAMs used for bioactivity and bioavailability and discussed areas of commonality and differences in the approaches taken, as well as exploring approaches to classification of chemicals and integration of bioactivity and bioavailability information.

Following the workshop, the team identified three key areas, and 3 teams were formed to explore in greater depth the work done so far and to maintain the spirit of co-creation that defines the Designathon:

- Chemical space: Explore chemical space and chemical uses, enrich the existing output with additional chemical information and further explore the results/classifications obtained with the reference chemicals to date.
- Biological space: Focus on biological space by modes of action (including, but not limited to, AOP networks) and mechanistic relevance assessment of the TK and TD methods proposed.
- Classification strategies: explore a future NAM-based approach to classification that would be tiered, as different types of tiered strategies were described.

The “co-creation phase” started in October 2024 and is likely to be concluded in Q1 2026. The focus now is to propose 2-3 classification approaches of human systemic toxicity and understand how these different approaches could be further evaluated in a “Testing Phase” and subsequent regulatory implementation.

Presentation: Oral



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Experiences in human health and the environment safety assessment education and training in Latin America: MAPEF workshops and SETAC Latin America NAMs interest group

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MAPEF Workshops. At the beginning of 2019, a group of people interested in alternative methods to animal testing, comprised of representatives from academia, government, and industry, met and established a working group with regular meetings. The focus is on pesticide regulation, and the organizers are from different sectors: Laboratorio de Métodos Alternativos – CONICET (National Research Council of Argentina), the National Service for Agrifood Health and Quality (SENASA), Atanor S.C.A./Albaugh, and researchers from the School of Agronomy of the University of Buenos Aires. We established an annual workshop called MAPEF (Alternative Methods for Agrochemical Evaluation) with the main objective of providing a space for updating and disseminating advances in the development of new alternative methodologies to the use of experimental animals, promoting acceptance, and creating an environment for exchange between industry, academia, and regulatory agencies. The first workshop in 2019 was in person in Buenos Aires, Argentina, but from 2020 to 2024, it has been held virtually since many of the participants are from other Latin American countries and

we had the opportunity of inviting leading experts from countries outside of Latin America. Speakers from the United States Environmental Protection Agency (EPA), European Food Safety Authority (EFSA), the European Commission's Joint Research Centre (JRC), the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), the Australian Pesticides and Veterinary Medicines Authority (APVMA), the Brazilian Health Regulatory Agency (ANVISA), among others, participated. The MAPEF Workshop attracts more than 700 participants each year from Argentina, Germany, Bolivia, Brazil, Costa Rica, Chile, Colombia, Cuba, Ecuador, the United States of America, Guatemala, Panama, Mexico, Paraguay, Peru, Uruguay, and Venezuela.

SETAC LA NAMs Interest Group. During the SETAC LA XV Meeting in 2023, a group of members proposed different activities related to NAMs. The first one was a training course focus on NAMs: "Implementation of alternative methods to the use of laboratory animals in safety studies of products for human use and agrochemicals". Additionally, a session was proposed and approved to provide a space for updating and disseminating advances in the development of new methodological approaches (NAMs). It also aims to promote the acceptance of new methodological approaches and generate an environment for exchange between industry, academia, and regulatory bodies. There was great interest in the roundtable with oral presentations and posters from different countries of Latin America. And last activity during the Congress, was the kickoff of the SETAC Latin America NAMs working group. It was a meeting with 14 participants from different sectors and countries. During 2024, this Working Group continued working with monthly meetings chaired by Ana Cione and Juan Ignacio Pina. We have done a survey within SETAC LA members to find out the interests and expertise within the region and developed a working plan to promote NAMs in Latin America.

These experiences reflect the need to carry on different initiatives in Latin America considering the specificity and needs of this region. These activities promoted the exchange of knowledge and experiences between experts of different regions and institutions and between experts of Latin America from different sectors in order to advance NAMs in the region.

Presentation: Oral



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Survey about NAMs within SETAC Latin America members

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During the SETAC Latin America XV Meeting in 2023, a group of members proposed different activities related to NAMs. The first one was a training course focus on NAMs: "Implementation of alternative methods to the use of laboratory animals in safety studies of products for human use and agrochemicals". Additionally, a session was proposed and approved to provide a space for updating and disseminating advances in the development of new methodological approaches (NAMs). It also aims to promote the acceptance of new methodological approaches and generate an environment for exchange between industry, academia, and regulatory bodies. There was great interest in the roundtable with oral presentations and posters from different countries of Latin America. And last activity during the Congress, was the kickoff of the SETAC Latin America NAMs working group. It was a meeting with 14 participants from different sectors and countries of Latin America. During 2024, this Working Group continued working with monthly meetings chaired by Ana Cione and Juan Ignacio Pina. We have done a survey within SETAC LA members to know about the interests and expertise within the region and developed a working plan to promote NAMs in Latin America. This survey was focused in two areas: 1. Challenges with adopting NAMs in Latin America, and 2. Information about the member. Regarding challenges, the questions were the challenges hindering the development and acceptance of NAMs in LA, and which specific actions can take SETAC LA to foster the advancement of NAMs. Regarding personal information, we asked about career stage, country of residence, and affiliation/sector. The results of the first question with 53 answers showed that the a. Lack of clarity regarding regulatory acceptance and b. Limitations of certified laboratories to run the regulatory studies are the major challenges with more than 60% and followed by c. Scientific state of art in science, with around 40%. The results of the second question were open comments with 38 answers. Regarding the second group of questions, with 49 answers, 40% were professionals with more than 24 years of experience, around 30% were professionals between 12 and 24 years of experience and 19% were professionals with less than 12 years of experience; only 8% were students. These experiences reflect the need to carry on different initiatives in Latin America considering the specificity and needs of this region. These activities promoted the exchange of knowledge and experiences between experts of different regions and institutions and between experts of Latin America from different sectors in order to advance NAMs in the region. The members that responded were from Brazil, Chile, Colombia, India, Nigeria, USA, Costa Rica, Argentina, Korea, and China. Finally, regarding affiliation, most of the answers came from representatives of academia (more than 60%), followed by business (25%), others (8.16%) and government (4%).

This survey is an important input for SETAC Latin America NAMS WG to plan their activities according to a diagnosis than by their own members, and a tool to use in the future to continue carry on these initiatives.

Presentation: Oral

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Brazilian law and regulations on animal use in science and education

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In Latin America, Brazil has played an important role in scientific research and now is putting robust and progressive efforts to implement alternative methods, in a forefront position related to the 3Rs principles. Laws and regulations have been proposed to ensure animal welfare and to prevent cruelty, such as Law n. 9,605 / February 12, 1998. It states in its Article 32, that it is a crime to commit acts of abuse or mistreatment to animals. But there was no specific law related to the use of animals in experiments or teaching in Brazil. Finally, the Law 11,794 of October 8, 2008 was passed in the Brazilian Congress and criteria for these purposes were established. The National Council on the Control of Animal Experiments (Concea) was created. Concea establishes rigorous requirements for the use of animals in scientific and educational projects, where each institution conducting animal experiments must have a duly accredited Animal Ethics Committee (CEUA). These committees are responsible for a detailed analysis of all research protocols, evaluating ethical and methodological aspects, verifying the application of the 3Rs Principles and ensuring that no alternative methods are available to replace animals in the analyzed protocol. Institutions are required to be registered in the Institutional Animal Use Registration (CIUCA) and submit a comprehensive annual report containing the total number of animals used, the species, the research purpose, and the procedures performed. Concea had developed specific guidelines for different taxonomic groups, with detailed protocols for mammals, rodents, non-human primates, and other animals, establishing rigorous standards for breeding, maintenance, and animal welfare. The Brazilian Directive on the Use and Management of Laboratory Animals addresses animal care during its use and handling. The Directive prohibits unnecessary duplication of experiments, requires the minimization of animal suffering, and demands the technical qualification of animal users. Concea is putting all its efforts into replacing the use of animals and has already recognized more than 40 alternative methods that refine, reduce or replace animals for testing. Concea Normative Regulations establish a five-year deadline for the mandatory replacement of traditional methods by recognized alternative ones, regardless of whether it is a cosmetic, agrochemical, or biomedical test. Concea Normative Regulation 58 took a pioneering step by prohibiting the use of animals in testing, developing, and controlling personal hygiene products, cosmetics, and perfumes that use ingredients or compounds with scientifically proven safety and efficacy in their formulations. In respect to the replacement of animals in education, Concea has a repository of alternative methods for education on its website and encourages students and teachers to use them, prohibiting the use of animals in classes where the purpose is merely demonstrative. In Brazil, there is a channel, Fala BR, where any Brazilian citizen can make complaints, even anonymously. Concea encourages people to access it and use it to report any case of cruelty to animals used in experiments. All these actions demonstrate Concea's concern and commitment to working towards ethical and high-quality research, always aiming to maximize the replacement of animal use.

Presentation: Oral



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Human brain microphysiological systems reveal synaptic disruptions from metal mixture exposure

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Toxic metal contaminants such as arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb) remain pervasive in environments with a history of industrial activity, posing significant risks to neurodevelopment. Early-life exposure to these metals has been linked to adverse neurological outcomes, particularly through disruptions in excitatory neuronal signaling. However, conventional animal models are limited in their ability to capture human-specific neurodevelopmental processes and the interactive effects of complex environmental mixtures. There is an urgent need for alternative methods that better reflect human neurotoxicity mechanisms. Here, we employ a human brain microphysiological system (bMPS) to investigate the impact of individual and combined metal exposures on synaptic structure, function, and neuronal excitability.

To model the developing human brain, neuroprogenitor cells were differentiated into 3D brain organoids containing neurons, astrocytes, and oligodendrocytes and were exposed to low-dose (environmentally relevant) and no-cytotoxic concentrations of As (100 nM), Cd (32 nM), Cr (100 nM), or Pb (320 nM) individually or as a mixture. Exposure occurred over a 4-week period during early (weeks 0-4) or late (weeks 8-12) differentiation. Synaptic integrity was assessed through immunostaining of pre- and post-synaptic markers (SYP, VGLUT1, PSD-95, Homer1) with colocalization analysis in SynapseJ. Neurite outgrowth was quantified via Sholl analysis, while functional neuronal activity was recorded using calcium imaging, with spike metrics analyzed in Python. RNA sequencing was performed to identify transcriptomic alterations, and theoretical additivity calculations were applied to assess whether metal mixture effects deviated from expected additive toxicity.

Results showed reductions in synaptic intensity density across all exposure conditions, with Cd, Cr, and the metal mixture causing the most pronounced disruptions in complete synaptic puncta. While pre- and post-synaptic contacts were preserved, complete synapses were reduced by 29-78%, indicating impaired synaptic protein distribution. Neurite outgrowth remained largely unaffected, suggesting that the observed synaptic deficits were not due to axonal or dendritic growth impairments. Electrophysiological assessments revealed that Cd exposure increased neuronal firing rates, while Cr exposure resulted in a dampened excitability response, demonstrating compound-specific neurotoxic effects. When analyzing mixture exposure, most endpoints showed a less-than-additive effect, with the exception of peak amplitude, which exhibited a slightly above-additive response. Transcriptomic profiling did not indicate major developmental trajectory disruptions but suggested subtle post-transcriptional modifications contributing to synaptic impairment.

These findings highlight the complexity of metal-induced neurotoxicity and the necessity of human-based models for studying subtle and mixture-specific effects. The human bMPS effectively captured synaptic alterations and functional disruptions that traditional animal models often fail

to predict. This work exemplifies the need to transition toward human-relevant approaches in neurotoxicology, replacing animal-based testing with models that more accurately reflect human physiology and environmental exposures.

Presentation: Oral

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From fish to frogs: Interspecies correlation estimation model to predict chronic aquatic amphibian toxicity from fish ELS endpoints

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Numerous studies show that acute fish toxicity data can predict acute toxicity of pesticides to aquatic life stages of amphibians. However, comparison of longer-term toxicity between the two groups has been limited due to a lack of relevant amphibian data. We investigated the relative chronic sensitivity of *Xenopus laevis*, investigated in amphibian metamorphosis assays (AMAs), for 87 pesticides (33 herbicides, 28 insecticides, 26 fungicides) compared to various fish species in the fish Early Life Stage (ELS) test. Comparisons were based on metrics for survival, body weight and length, which were extracted from publicly available registration documents (i.e., EU or US regulatory authorities) or, in a few cases, from unpublished study reports. We assessed the statistical correlation using Pearson's correlation coefficient and linear regression, aiming to derive an interspecies correlation estimation (ICE) model to predict chronic aquatic amphibian toxicity from fish ELS endpoints. In an initial analysis, the AMA was found to be equally (within a factor of 3) or less sensitive than the fish ELS test for most pesticides (84%). Paired t-tests identified the ELS as significantly more sensitive than the AMA for all investigated metrics ($p < 0.05$). Strong and statistically significant correlations ($rP \geq 0.78$) between endpoints of the two test systems were observed across all parameters and pesticide classes. Although the test design of the AMA is not suited for deriving No Observed Effect Concentration values for quantitative use in an environmental risk assessment (ERA), the dataset seems suitable to derive an ICE model to support the use of fish as a surrogate species for aquatic phase amphibians. This approach would minimize the need for additional vertebrate studies in the ERA of plant protection products.

Presentation: Oral



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A step toward an integrated testing strategy for acute oral toxicity assessment of pesticides

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Acute oral toxicity represents a global regulatory requirement for certain products registration, such as pesticides. Typically, standardized tests use rodents as the testing species and the LD50 (mg/kg), dose expected to produce lethality in 50% of the population, as the endpoint to determine the hazard category as well as the warning labeling of a product.

Despite considerable efforts have been made to replace rodent assays, it remains a challenge. Different non-animal-based approaches have been developed to reduce animals use such as: (1) *in vitro* cytotoxicity assays to estimate the initial dose for an *in vivo* assay. (2) the GHS (Globally Harmonized System) Additive Formula (AF) to estimate the acute toxicity of mixtures, based on the concentration and the toxicity of each ingredient. Though, reliable toxicological data for individual components might be limited available and the variability between LD50 values, from multiple reports for one component, must be acceptable to ensure robust interpretation. A possible solution to this would be an AF based on LD50 values estimated by QSAR (quantitative structure-activity relationship) models. For example, the CATMoS (Collaborative Acute Toxicity Modeling Suite), developed through an international collaboration led by the ICCVAM (U.S. Interagency Coordinating Committee on the Validation of Alternative Methods), was designed to predict the LD50 values of unknown substances.

Our goal was to establish a first step on the development of an integrated testing strategy for acute oral toxicity assessment of pesticides, composed of an *in vitro* cytotoxicity assay and the AF. We tested 22 pesticide formulations supplied by ATANOR S.C.A and Gleba S.C.A along with their associated *vivo* data. The BALB/3T3 Neutral-Red Uptake Cytotoxicity Assay (OECD TG 129) was used to estimate the *in vitro* LD50 values and the AF was applied, using active ingredients concentrations and their LD50 values, predicted by the CaTMoS from the ICE (Integrated Chemical Environment) web server (<https://ice.ntp.niehs.nih.gov/>). Then, formulations were classified as potentially safe or potentially toxic (LD50 cutoff value of 2000 mg/kg) for the *in vitro* and AF datasets. The performance metrics of both methods (accuracy, sensitivity, specificity, overestimation rate and underestimation rate) were calculated relative to the *in vivo* data.

Accuracy was similar (*in vitro*: 56% and FA: 62%) for both but the cytotoxicity assay was more efficient in estimating potentially toxic pesticides (sensitivity *in vitro*: 100% and sensitivity AF: 60%) while the AF was better at identifying potentially safe formulations (specificity *in*

vitro: 13% and specificity AF: 65%). Additionally, the overestimation rate was greater with the *in vitro* method (70%) and underestimation rate was relevant with the FA (9%).

Considering the cost-time relationship between both methods, a potential future strategy could be to initially use the AF to estimate potentially safe formulations, followed by the cytotoxicity test to confirm potentially toxic pesticides. It would be necessary to determine the appropriate course of action if classifications from both methods do not align. Additionally, evaluating the cellular mechanisms contributing to acute lethality and the toxicokinetics of the active ingredients in the formulations could be added as supporting evidence.

Presentation: Oral

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Utilization of human on a chip systems for rare disease regulatory submissions

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One of the primary limitations in drug discovery and toxicology research is the lack of good model systems between the single cell level and animal or human systems. This is especially true for neurodegenerative diseases such as ALS, Myasthenia Gravis, Charcot-Marie-Tooth Disease and Alzheimer's as well as for oncology. In addition, with the banning of animals for toxicology testing in many industries body-on-a-chip systems to replace animals with human mimics is essential for product development and safety testing. Our research focus is on the establishment of functional *in vitro* systems to address this deficit where we seek to create organs and subsystems to model motor control, muscle function, myelination and cognitive function, as well as cardiac and liver subsystems. The idea is to integrate microsystems fabrication technology and surface modifications with protein and cellular components, for initiating and maintaining self-assembly and growth into biologically, mechanically and electronically interactive functional multi-component systems. Specific embodiments of this technology are the creation of a functional human NMJ system to understand ALS and a long-term potentiation (LTP) for AD utilizing clinically relevant functional readouts. The models have demonstrated the LTP model can reproduce A β pathology and tauopathy in human cortical neurons and demonstrated how AD therapeutics can recover these effects. We have also developed models of toxicity and demonstrated an oncology model when we evaluated efficacy and off target toxicity for both parent compound and its metabolites. Examples will be given of some of the other human-on-a-chip systems being developed for CNS and PNS disease applications as well as the results Sanofi has used as efficacy data from one of our models to file the first IND only from MPS data that has enabled a clinical trial (#NCT04658472) that has now proceeded to a two-arm Phase III (NCT06290141 and NCT06290128).

Presentation: Oral



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MyCalPharm: Simulated animal experiments for students of pharmacology

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Animal experiments were traditionally carried out during practical hours to teach drug actions to pharmacology students. In the 1990s, a movement to replace animals in teaching started in India. For more than thirty years, the authors created, distributed, and used computer-simulated animal experiments for medical and pharmacy students in India. Their software packages have helped policymakers, regulatory agencies, and colleges replace animals in practical pharmacology classes and save thousands of animals in India.

Recently, a new package titled MyCalPharm (www.mycalpharm.com) has been released. It consists of 37 programs simulating animal experiments grouped into two sections, namely Xpharm and Xcology, which target medical students and pharmacy students, respectively. Each program, when run, displays the experimental setup and allows the user to interact with it through the mouse and the keyboard. The user can choose a drug from the list displayed and administer the same. The drug action is shown on the screen in animated sequences resembling a realistic experiment. The user can modify the dose to witness the dose-dependent effect. All programs (experiments) are provided with adequate text material and videos for some experiments to learn about the theoretical and dissection aspects of the experiment for better understanding. All the actions/responses/inputs of the user are automatically stored in a log file. The user can collect data, enter it in the tables provided, and store it. At the end of the experiment, the user is asked to answer a few questions. Once the experiment is completed, all data, including the logs, graphs (if generated), and answers, can be stored in PDF format and/or printed out. The file can be mailed to the teacher.

Each experiment can be run in three modes: (a) Tutorial, (b) Examination Practice, and (c) Examination modes. Once the student is familiar with the experiment after running the program in tutorial mode, he/she can practice examination mode. In the examination mode, tasks are assigned to students who should find out the nature of the unidentified drugs using their knowledge gained by running the tutorial mode. They can check their results by asking the program to reveal the unknown drugs at the end of the experiment.

The examination mode is fully password-protected and meant for teachers to conduct examinations for students. The students should perform the experiment properly and carry out the tasks to find the answers. The unknown drugs can be revealed only to the teachers. A copy of the entire experiment is stored for the teacher to evaluate it.

The software is user-friendly and realistic. It is widely used in medical and pharmacy colleges in India to replace animals without compromising the quality of teaching for medical and pharmacy undergraduates.

Presentation: Oral

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A guidance towards drafting retrospective assessment results for scientific projects involving non-human primates and severe suffering

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According to the Directive 2010/63/EU, all projects using non-human primates and projects involving procedures classified as severe, shall undergo a retrospective assessment [1].

These shall evaluate whether:

- the objectives of the project were achieved,
- the harm inflicted on the animals involved (including species and numbers),
- the severity of the procedures,
- and any elements that may contribute to the further implementation of the 3Rs.

Thus, retrospective assessment is considered an extremely powerful tool to facilitate critical review of the use of animals in scientific procedures, to identify potential 3R improvements for future projects and, if published, to enhance transparency to the public [2]. For example, if a retrospective assessment reveals that certain experimental trials do not deliver the promised results or that the harm inflicted on the animals is regularly underestimated, it can be decided accordingly that such trials are not expedient, and follow-up projects should not be approved. In addition, if a project resulted in surplus animals (animals bred for but not used in procedures), learnings from this project could allow reducing the number of surplus animals in similar projects in the future. Therefore, this information must also be publicly available to enable the Directive's objective of enhancing transparency and improvements of the 3Rs. The ALURES database publishes non-technical project summaries as well as summaries of the results of retrospective assessments [3]. Since harmonization in the reporting of retrospective assessment results (RAR) across EU member states is essential for adequate information of the public, the European Commission published an official guidance document in 2024 [4].

Here, we discuss this guidance document, which aims to aid competent authorities to draft meaningful, clear and succinct summaries of the RAR. This document was developed by the National Contact Points from the EU member states with the participation of stakeholders i. a. from academia, regulatory and animal welfare. In addition, illustrative examples of RAR including quality ratings are displayed as well [4].

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Presentation: Oral

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Expanding the reach of animal-free science: The role of accessible and inclusive informal education in advancing human-relevant research

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Traditional academic education often struggles to keep pace with advancements in non-animal research methods. To bridge this gap, accessible and inclusive informal education – such as webinars and workshops – plays a critical role in complementing formal education and accelerating the adoption of animal-free New Approach Methodologies (NAMs). These interdisciplinary learning opportunities equip researchers, educators, policymakers, and industry professionals with the knowledge and tools needed to transition toward human-centered research. Beyond educating professionals, these initiatives are freely available to a broad audience, ensuring that anyone – regardless of background or

expertise – can engage with and advocate for humane, innovative science. By making cutting-edge research and ethical considerations accessible to all, these programs empower citizens to push for political change, as an informed public is essential for transforming the current paradigm of animal research.

One such initiative is CAAT's free, bimonthly 3Rs Training Webinar series, which provides a comprehensive overview of NAMs and their potential to reduce and replace animal experimentation. Recent topics have included organ-on-a-chip technology, non-invasive wildlife research techniques, xenofree 3D bioprinting, biases favoring animal studies in scientific review processes, and the moral injury experienced by laboratory animal workers. To further support early-career scientists, CAAT and the Physicians Committee for Responsible Medicine offer the Animal-Free Workshop Series for Early-Career Researchers, a free and globally accessible online program designed to help emerging scientists explore non-animal research methods while building essential career skills. Speakers across both programs include biomedical scientists, toxicologists, veterinarians, physicians, environmental engineers, bioethicists, sociologists, and historians – highlighting the interdisciplinary nature of this paradigm shift in science.

Despite the well-documented advantages of animal-free NAMs – such as improved translatability to human biology and the elimination of animal suffering – progress toward replacing animal models remains slow. A key focus of these initiatives is bridging the gap between scientific evidence and regulatory acceptance, encouraging critical thinking about the status quo, and helping attendees understand the structural and cultural barriers to change.

This presentation will showcase how accessible and inclusive informal education fosters interdisciplinary collaboration, challenges biases favoring animal-based studies, and facilitates knowledge exchange among diverse stakeholders. Attendees will gain practical strategies for designing and delivering impactful webinars and workshops that engage diverse audiences, drive scientific progress, and reduce reliance on animal models.

Presentation: Oral



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A review of the role of animal sentience recognition in law & policy

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The issue of animal sentience has been of increasing interest in science, law, and animal advocacy. Scientists have begun to provide data-driven support for much of what animal advocates have long intuited – non-human animals feel pain and pleasure. Subsequently, some jurisdictions are acknowledging animal sentience through legal means in the hopes of elevating the interests of non-human animals under the law. Today, some form of non-human animal sentience is recognized in at least thirty jurisdictions. Yet, the practical implications of this legal trend remain unclear.

First, there is ambiguity in the meaning of “sentience” as used across jurisdictions. For example, Oregon law implies that sentience is the ability to feel pain and pleasure while Quebec law implies sentience is synonymous with “having biological needs.” Still other jurisdictions omit a definition or explanation of the word all together.

Second, a legal declaration of sentience does not mandate change in the way animals are treated. In many ways, these types of laws are symbolic in nature. But symbolic does not mean futile. The declaration of animals as sentient, whether through legal means or by scientific experts, is a form of virtue signaling not unlike labeling elephants and chimpanzees as “intelligent.” Embedded with claims of sentience is the sentiment that “they are like us.”

Presentation: Oral

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No one-size-fits-all: The challenges of global risk assessment

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The journey towards achieving global harmonization in risk assessment is riddled with obstacles, stemming from varied regulatory frameworks, inconsistent hazard classifications, and differing views on animal testing across different sectors and regions. This presentation will explore the intricate challenges of aligning risk assessments amidst these disparities, with a particular emphasis on the cosmetic industry.

The European Union’s ban on animal testing for cosmetics, as mandated by the Cosmetic Products Regulation (CPR), presents both benefits and inconsistencies. For example, while the ban prohibits domestic animal testing, it also forces the exclusion of *in vivo* data from non-EU countries, creating a fragmented regulatory landscape. Additionally, the definition of cosmetics is not harmonized across sectors, leading to complexities in regulatory compliance. The scope of the ban on animal testing also varies between sectors, further complicating efforts to achieve a unified approach.

Drawing on the extensive work of Cosmetics Europe, the presentation will focus on one particular regulatory dilemma for the cosmetic sector, which is worker safety assessment. In fact, despite the progress made, the REACH regulation continues to mandate animal tests for cosmetic-only ingredients to assess worker safety, undermining the positive impact of the CPR’s animal testing ban.

The talk will underscore the need for a coordinated effort to align regulatory requirements across regions and sectors and will provide a platform for critically examining the barriers to harmonization while exploring innovative solutions that respect regulatory diversity while advancing the global commitment to ethical science and sustainability.

Presentation: Oral



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Integration of non-animal approaches, weight of evidence and uncertainty analysis in hazard characterization and risk assessment of agrochemicals

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This presentation will provide an overview of real case regulatory implementation recent examples in the pesticides area.

Depending on regulatory requirements, extensive databases of toxicological data are generated for agrochemicals active substance, which allow deriving key information to characterize hazard, dose response and set Point of Departure (PoDs) to conduct solid human health risk assessments. Standard toxicology assays are mostly *in vivo* methods conducted according to internationally agreed test guidelines and Good Laboratory Practice Standards to characterize key features of toxicity (i.e., adversity) and life stage sensitivity. However, increasingly often, tailored hypothesis-based studies are conducted to characterize Mode of Action (MoA) and Human Relevance Framework (HRF) of certain toxicological findings using bespoke assays. These assays will often have only a “fit for purpose” validation and they will help characterize key events within an Adverse Outcome Pathway (not necessarily adversity), sometimes with quantitative relationship established within the context of a Mode of Action program. In all cases, all the available lines of evidence are then assembled using relevant Weight of Evidence and Uncertainty analysis frameworks, to derive scientific conclusions, where relevant classification and labeling conclusions, and finally set human relevant points of departure for risk assessment with related uncertainty factors.

As case examples specific to this presentation we will review a case example of weight of evidence/uncertainty analysis for the case of a naturally occurring insecticide, Spinosad, where the human relevance of certain parturition effects associated to high dose treatment in two generation rat reproduction toxicity studies was investigated using Integrated Approaches to Testing and Assessment (IATA) principles and WHO MOA/HRP frameworks, generating evidence with a combination of NAMs (covering *ex vivo* studies, comparative *in vitro* kinetic studies and PBK) and finally assessing relevance for reproductive toxicity classification and labeling in EU and for risk assessment to human health in the US.

Presentation: Oral

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New approach methodologies for estimating chronic aquatic toxicity of cosmetic ingredients: A comparison of acute-to-chronic ratios and *in silico* tools

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The cosmetic industry is increasingly committed to sustainable product development, with a particular focus on reducing the environmental footprint of cosmetic formulations. A key component of this effort lies in assessing the potential aquatic toxicity of cosmetic ingredients early in the development process. However, the ban on animal testing poses a significant challenge in acquiring chronic aquatic toxicity data, which is critical for conducting long-term environmental risk assessments. This study seeks to address this gap by evaluating New Approach Methodologies (NAMs), including acute-to-chronic ratios (ACRs) and *in silico* QSAR tools, as alternative methods for estimating chronic aquatic toxicity. We used ACRs, numerical factors obtained from various sources, which extrapolate acute into chronic toxicity data. The mode of action of each substance was predicted using Toxtree software and included in the ACR analysis. The chronic toxicity NOEC (No Observed Effect Concentration) values generated by these methods for algae, daphnids, and fish were compared to experimental data obtained from the European Chemicals Agency (ECHA) database. Such data comprised results from standardized OECD tests on 41 cosmetic ingredients with well-defined molecular structures and reliable acute and chronic ecotoxicity data. Comparisons between extrapolated / predicted NOECs and experimental data were based on regression analysis, fold-difference, and accuracy percentage in the ingredients classification based on the Globally Harmonized System (GHS) using the generated NOECs. The results indicate that ACRs can provide generally accurate NOECs, as such values exhibited a statistically significant correlation with experimental values ($R^2 = 0.65-0.87$; $p < 0.001$) and, in most cases, less than 5-fold different from experimental values (up to 97.3% for algae, 50% for daphnids and 66.7% for fish). Also, accurate classifications based on GHS (up to 94.6% for algae, 86.4% for daphnids and 75% for fish) were generated. According to our data, predictions generated using different ACR values provided the most reliable NOECs using lower ACR values (4.07 for algae, 5.53 for daphnids, and 10 for fish), while a higher extrapolation factor (100) led to less accurate extrapolated NOECs across all three organism groups. ACRs based on substance mode of action also provided reliable NOECs. On the other hand, the *in silico* tools employed to



predict NOECs demonstrated poor correlation with experimental data, with differences up to 10-fold for several substances (37.1%-63.6%). Our findings suggest that ACRs represent a promising tool for screening-level assessments in cases where chronic aquatic toxicity data are limited. Additionally, *in silico* tools hold significant potential as innovative alternatives. However, further studies using a broader range of tools are needed to refine their accuracy and reliability. Continued research in this area will enhance the applicability of these methods, supporting the cosmetic industry's commitment to innovation, regulatory compliance, and environmental sustainability.

Presentation: Oral

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Toward a negative list – Eliminating high-severity animal models lacking translatability

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Many high-severity animal models continue to be used in biomedical research despite their failure to translate to human health benefits. While eliminating ineffective models is not a new concept, a structured “negative list” – explicitly identifying high-severity models with poor translatability – is urgently needed. This talk presents specific examples of such models, demonstrating why they should be discontinued and replaced with human-relevant methods that improve research outcomes while preventing needless animal suffering.

A key example is nerve regeneration research, where animal models have consistently failed to predict human outcomes. Despite decades

of spinal cord injury research in animals, their translatability remains extremely poor. While many experimental treatments show success in preclinical studies, the vast majority fail in human clinical trials due to fundamental anatomical and physiological differences. Meanwhile, 3D bioprinting, microfluidic nerve-on-a-chip platforms, and engineered human neural tissue scaffolds provide more predictive, ethical, and scalable alternatives. Similarly, neurodegenerative disease research relies on genetically modified rodents and non-human primates to model Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Yet, no animal model accurately replicates the complex pathology of these human-specific disorders. The failure rate for Alzheimer's drug candidates is 99.6%, underscoring the disconnect between preclinical findings and human outcomes. These models involve toxin-induced lesions, artificial genetic modifications, and forced behavioral testing, leading to paralysis, weight loss, and severe neurological impairment, without capturing the progressive nature of human neurodegeneration. Meanwhile, patient-derived induced pluripotent stem cells, human brain organoids, and organ-on-chip models provide human-specific insights into disease mechanisms, rendering continued reliance on these suffering-intensive animal models unjustifiable. Other high-severity experiments that should be included in a negative list include, among many others, pain studies, trauma models, sexual and physical violence studies, forced swim and immobilization stress tests, sepsis models, irradiation studies, and metastatic cancer models.

By identifying and eliminating high-severity, low-translatability models, biomedical research can reduce needless animal suffering while improving human health outcomes. This presentation will discuss the scientific and regulatory steps needed to implement a negative list, emphasizing harm-benefit assessments, policy reforms, and interdisciplinary collaboration. By adopting human-relevant research technologies, we can replace outdated, ethically unjustifiable animal models with methods that truly advance medical science.

Presentation: Oral



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The intersection of cosmetics and chemical safety: Lessons for progress

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The International Collaboration on Cosmetics Safety (ICCS – launched in Feb 2023) is focused on advancing the global adoption of animal-free science for human and environmental safety assessments of cosmetic products and their ingredients through funding scientific research, education and training activities, and regulatory engagement.

While animal tests on cosmetics are increasingly banned around the globe, there remain chemical regulatory requirements in place in many regions that still mandate animal tests and prevent regulatory use of animal-free new approach methodologies (NAMs). This can often result in regulatory requests for animal test data to fulfil chemical regulations when the same or similar information requirement can be met using animal-free NAMs under the cosmetic regulation (e.g., EU: REACH worker safety vs Cosmetic Product Regulation (CPR) consumer safety requirements).

ICCS is working with all stakeholders involved in the regulation of cosmetic products and ingredients to share learnings from applying NAMs and Next Generation Risk Assessment (NGRA) frameworks to ensure cosmetic safety and identifying opportunities to advance regulatory use of NAMs/NGRA approaches for chemical safety. ICCS aims to accelerate global regulatory adoption of animal-free safety science for cosmetic products & ingredients and, in so doing, help standardize global regulatory information requirements for cosmetics to avoid the need for redundant/unnecessary testing.

Presentation: Oral

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Ensuring the credibility of new approach methodologies in regulatory toxicology: The vital role of validation and ring trials

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The validation of new approach methodologies (NAMs) is a cornerstone for their acceptance and integration into regulatory toxicology. This talk delineates the decisive aspects of relevance and reliability in the validation process, highlighting the indispensable role of ring trials. Relevance pertains to the extent to which NAMs accurately measure or predict the biological effect of interest, ensuring that the data generated are pertinent to hazard and risk assessment. Reliability, on the other hand, involves the consistency of NAMs' performance across different laboratories and conditions, which is crucial for their widespread acceptance. Jacobs et al. (2024) discuss the role of ring trials in avoiding a reproducibility crisis in regulatory toxicology, highlighting the need for robust validation frameworks and the necessity of reliable and relevant data for the acceptance of NAMs in regulatory contexts.

The success of validating a method critically depends on how well the method has been developed and how well it is used. The OECD's Good *In vitro* Method Practices (GIVIMP) guidance document 286 provides a structured framework for the development, validation and implementation of *in vitro* methods. GIVIMP addresses various elements such as materials, reagents, test systems, and standard operating procedures, ensuring that the methods are robust and reproducible. The guidance also emphasizes the importance of quality assurance and control measures, which are integral to the validation process.

The transition from traditional animal-based testing to NAMs represents a significant shift in regulatory toxicology. This transition is driven by the need for more human-relevant data, advancements in science and technology, and ethical considerations. However, the success of this transition hinges on meticulous development and the rigorous validation of NAMs to ensure that they can reliably predict human health outcomes. The integration of NAMs into regulatory frameworks requires a concerted effort to standardize validation processes, implement ring trials, and adhere to guidelines such as GIVIMP.

Reference

[1] Jacobs, M. N. et al. (2024). *Arch Toxicol* 98, 2047-2063.

Presentation: Oral



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Actions for paradigm shifts: Ethical considerations about severe suffering and strategies to refine and regulate

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The 3Rs principles contribute significantly to reducing suffering inflicted on animals. However, one major issue that is not addressed by this framework concerns cases in which the scientific goal requires a higher degree of harm. To put it differently, the refinement principle is limited by the research goal. Brazilian regulations include exception mechanisms that allow severe suffering to remain unalleviated on scientific grounds. For example, this includes withholding analgesia. We propose that setting stronger regulatory limits for highly invasive experiments is necessary in Brazil for both ethical and scientific purposes. First, animals in Brazil are protected by a set of regulations that acknowledge their dignity. This means they have the right not to be subjected to cruelty and mistreatment. Moreover, a recent qualitative study conducted with veterinarians in Brazil suggests that evaluations of protocols for severe procedures can be influenced by social and political context. Nevertheless, local and international concerns regarding laboratory animal suffering combined with a growing demand for more solid justifications and adoption of refining measures have increased scrutiny over such practices. For instance, EU and the UK regulations set upper limits on harm, meaning that procedures in which suffering cannot be alleviated are not permitted. Taken together, these developments highlight the moral obligation of working towards reducing severe suffering in animal research. Therefore, more objective and strict criteria are necessary to determine whether a procedure should be performed without alleviating animal suffering in Brazil. Second, it is well known that animal health and welfare are key for scientific robustness and validity. However, empirical evidence in Brazil suggests housing animals in conventional systems is still a common reality, which increases susceptibility to infectious diseases outbreaks. Thus, we argue that procedures classified as severe should be restricted to facilities with higher sanitary and genetic standards. In summary, we argue for stricter regulatory limits to protect animals from severe procedures in Brazil. This would increase regulatory protection of lab animals and align Brazilian regulations with international standards. In exceptional cases where research involving infliction of severe harm can be morally and scientifically justified, we strongly advocate the need for the full application of refinements, humane endpoints and higher standards of husbandry and health.

Presentation: Oral

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NAM-based risk assessment into practice: The ASPA framework

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Next-generation risk assessment (NGRA) is considered a pivotal paradigm for integration of NAM-based data for toxicity, exposure assessment and kinetics, to enable regulatory decision-making whether a chemical is safe or not. To accelerate the use of NGRA approaches for regulatory decision-making, RISK-HUNT3R – in cooperation with the ASPIS cluster consisting of the three EU-funded projects RISK-HUNT3R, ONTOX, and PrecisionTox – has developed a modular workflow. This workflow, entitled the ASPIS-initiated Alternative Safety Profiling Algorithm (ASPA), provides structured guidance on both data generation and data interpretation and allows for parametrization of decision points and rule settings. Moreover, it strengthens the transparency and robustness of decision-making and reporting. ASPA is intended for different types of end-users: end-users that wish to compile a dossier and thus need to generate data, and end-users that need to evaluate a dossier and thus need to interpret data. ASPA is structured along three information pillars, namely information about hazard, the kinetic properties of a substance, and exposure. A selected set of case studies was conducted with the aim to test, evaluate and refine ASPA as well as to determine the applicability domain for the use of ASPA and its different building blocks. In this presentation, the latest version of ASPA that was evaluated by a larger group of experts from regulatory authorities, industry and academia during a workshop hosted at the German Institute for Risk Assessment, will be presented. We will discuss its applicability as well as limitations of the workflow and therewith highlight areas for future research and improvement.

Presentation: Oral



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Advanced *in silico* methods for the prediction of genotoxicity

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In silico methods for the prediction of a variety of toxic endpoints have been developed for many years and have advanced beyond initial local (Q)SAR models and expert systems to incorporate diverse prediction strategies, including Read-Across and the use of Adverse Outcome Pathways (AOPs). These diverse methodologies are increasingly used to reduce, refine and replace animal assays, whether alone or in combination with *in vitro* assays.

Briefly, a (Q)SAR model uses a statistical approach to infer relationships between known toxicity data and chemical structures, whereas an expert system uses the knowledge from this data and additional sources such as mechanistic knowledge and inference from biological pathways to create structural alerts which reflect the toxicity of a class of compounds. These two methods are integrated together, with subsequent expert review, under ICH M7 for the prediction of *in vitro* mutagenicity of impurities, a key part of the management of these.

Expert systems and (Q)SARs are also extensively used, as part of defined approaches, for the prediction of skin sensitizing potency, especially in the context of cosmetics where for much of the world animal tests have been banned. These two use-cases have clearly defined outcomes and large training sets of robust data for the generation of these models and are the outcomes with the greatest regulatory acceptance.

Read-across can be argued as a local extension of an expert system; the use of a similar surrogate to define a safe level of another compound, using an understanding of reactivity, mechanism of action, DMPK and chemoinformatics to address the question of “what is similar”. It is becoming increasingly accepted; for example, in nitrosamine guidance and the recent EMA reflection paper on the qualification of non-mutagenic impurities.

AOPs curate knowledge about all of the Key Events that connect the initial interaction with a biomolecule, such as DNA alkylation, with an ultimate adverse outcome such as cancer. In the context of genotoxicity, this includes such steps as incomplete repair, the introduction of persistent mutation, cell immortalization and proliferation as a consequence of accumulated mutations. Regulatorily, these can be used to address the questions raised in the ICH S1b guidance to permit waiving of carcinogenicity studies for some APIs.

This talk will discuss these *in silico* methodologies, with examples, and illustrate opportunities for the further expansion of the *in silico* domain.

Presentation: Oral

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A review of the 3Rs, harm/benefit analysis, and the distinction between sentience, cognition, and self-awareness

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Despite important philosophical arguments and ethical considerations regarding the use of animals in scientific procedures, a grounding of animal research in bioethical principles (akin to globally accepted frameworks for research with human participants) does not exist in the legal system of many countries, including that of the United States. While the 3Rs are often incorporated in laws and policies that affect the care and use of animals in research, harm/benefit analysis does not universally guide our selection of the most appropriate animal species for a research project. Humans write laws that reflect both personal and societal beliefs and choices. Thus, the incorporation of sentience, cognition and/or self-awareness is not made or understood as a key ethical contributor to the legal approach to animal-based research. Using elements from the recent publication “Animal sentience should be the key for future legislation” by Margaret Landi and Lida Anestidou, this presentation will serve as the preamble to the subsequent talks by providing background on what the terms sentience, cognition and self-awareness mean in the context of established ethical elements such as the 3Rs and harm/benefit analysis.

Presentation: Oral



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Activities under the European Partnership for the Assessment of Risks from Chemicals (PARC) in support of the regulatory implementation of next-generation risk assessment (NGRA)

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Developing and implementing next-generation risk assessment (NGRA) frameworks as the default approach to human health (HH) and environmental (ENV) chemical risk assessment (CRA) requires a concerted effort by diverse actors from the chemical risk assessment (CRA) community. In this presentation we inform about ongoing work under the European Partnership for the Assessment of Risks from Chemicals (PARC; <https://eu-parc.eu>) to support this process.

In the first part we will present the activity NGRARoute, which aims at implementing Next-Generation HH/ENV Risk Assessment as the default approach in EU chemicals legislation. NGRARoute supports the European Commission's "Roadmap towards phasing out animal testing in chemical safety assessments", *inter alia* by developing the conceptual foundations for future NGRA frameworks/workflows or by engaging in activities for the concrete replacement of *in vivo* testing, e.g., for acute toxicity testing.

In the second part we demonstrate how our online knowledge management and community platform PARCopedia (<https://parcopedia.eu>) can help break down knowledge and language barriers between the diverse segments of the chemical risk assessment community, fostering the exchange of knowledge and enabling a joint effort for innovating chemical risk assessment and management.

Presentation: Oral

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The role model for DART: Lessons learned from DNT-IVB battery to guide a NAM-based DART roadmap

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Background and purpose: In 2014, the ISTNET Workshop in Zurich developed a roadmap for developmental neurotoxicity (DNT) testing using new approach methodologies (NAMs), culminating in OECD's 2023 recommendation (No. 377) for regulatory use of the DNT *in vitro* battery (IVB). Developmental and reproductive toxicity (DART) presents an even greater challenge, with thousands of untested chemicals and ethical concerns surrounding traditional animal models. A strategy is needed to assess DART potential efficiently.

Methods: The ISTNET-DART meeting (Zurich, September 12-13, 2024) gathered multi-stakeholder experts to build consensus on *in silico* and *in vitro* approaches for DART regulatory decisions. The goal was to develop a NAM-based integrated testing strategy (ITS) aligned with regulatory requirements. The ISTNET-DART meeting was followed up by a breakout meeting at the Animal Free-Chemical Safety Assessment Conference (AF-CSA conference, Brussels, March 5, 2025).

Results: The ISTNET-DART meeting brought together 61 participants from 12 countries. Discussions built on lessons from DNT-NAM development, covering reproductive and developmental biology, existing *in vitro* methods, PBK modeling, and regulatory perspectives on NAMs for DART testing. Insights included readiness, validation, and DART implementation in next-generation risk assessment (NGRA) and EU strategies for phasing out animal testing. A roadmap publication summarizing the findings will outline the path forward and call for further contributions to NAM readiness. Thereby, a six-step framework for legal implementation of NAMs from test development up to regulatory acceptance, which was developed for DNT, will guide DART NAM development. During the AF-CSA conference, further stakeholder alignment with regards to the "European Roadmap for phasing out animal testing for chemical safety assessments" was strived for.

Conclusion: The success of the DNT-IVB stemmed from early stakeholder involvement and a biology-driven approach. Applying this strategy to DART aims to achieve similar progress in regulatory acceptance and implementation of alternative methods by including multiple stakeholder views from academia, regulators, industry and the medical sector.

Presentation: Oral



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European partnership for alternative approaches to animal testing (EPAA): 20 years of 3Rs progress

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The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a unique partnership between the European Commission (DGs GROW, ENV, SANTE, JRC, RTD), European Regulatory Agencies (ECHA, EFSA, EMA), European Industry Federations (AISE, AHE, CEFIC, CE, CLE, EFPIA, FDE, IFRA, SMEUnited) and 39 companies to refine, reduce and replace regulatory animal testing.

EPAA was established in 2005 and for the last twenty years has funded a diverse range of collaborative scientific projects and annual Partner Forum events, all aiming to advance regulatory use of 3Rs through bringing together scientific experts from academia, commission, industry, member state authorities and regulatory agencies. EPAA communicates progress to stakeholders quarterly through our steering committee – mirror group meetings and annually through our Annual Congress event and European Parliament lunch debates.

In recent years the EPAA has re-focused its activities to align with and inform the European Commission Roadmap towards phasing out animal testing for chemical safety assessment through creating a series of platforms to co-create proposals on how to address strategic roadmap challenges:

- Use of New Approach Methodologies (NAMs) for Human Health Systemic Safety Assessment via EPAA NAM Designathon Challenge & EPAA NAM User Forum
- Use of NAMs for Environmental Safety Assessment, following the 2023 EPAA Partners Forum and new EPAA project on this topic
- Use of NAMs for the Assessment of Endocrine Disruption, via 2024 EPAA Partners Forum

In addition, EPAA has initiated new project activities to develop roadmap proposals to address priority regulatory needs on Use of NAMs for Acute Toxicity Assessment, Second-Species Sub-Chronic Toxicity Testing and implementation of a new paradigm for Carcinogenicity & Genotoxicity Assessment.

Finally, in March 2025, EPAA partnered with the European Commission to organize a multi-stakeholder conference on Animal-Free Chemical Safety Assessment (AF-CSA). The proposals developed during the AF-CSA conference will be considered for potential inclusion in the draft Commission Roadmap.

Presentation: Oral

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Non-animal reprotoxicity: Gender-specific analysis methods using male and female reproductive platforms

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Assessing reproductive and developmental toxicity is crucial for human health, identifying substances harmful to reproduction and fetal development. These toxicities cause infertility, congenital anomalies, and long-term risks. Traditional methods, like OECD TG 414, use animals, with ethical and scientific limitations, such as difficulty extrapolating results to humans, exemplified by the thalidomide disaster.

New Approach Methodologies (NAMs) emerge as alternatives, using *in vitro* systems, computational models, and high-throughput screening. NAMs improve accuracy and efficiency, creating human-relevant models, essential for studying complex processes like fertility and fetal growth. Using human cells and tissues generates more relevant data, accelerating tests and regulatory decisions.

In Brazil, advances like CONCEA Resolution No. 58 and the ban on animal-tested cosmetics highlight progress in NAMs. However, the lack of reliable NAMs for reproductive toxicity and teratogenicity limits innovation, especially for natural ingredients from Brazilian biodiversity.

Human-based models, such as ovarian and testicular spheroids, and *in vitro* placental barrier models, offer insights into reproductive toxicity and teratogenicity, simulating human exposure conditions. Combined with organ-equivalent models, like 3D skin constructs, they simulate exposure scenarios, accelerating regulatory decisions and promoting ethical research.

For Brazil, NAMs are strategic for the cosmetic industry, driven by clean beauty and natural ingredients. Robust NAMs enable global competitiveness, leading in innovation and sustainability.

Developing NAMs is essential to replace animal testing, enhancing *in vitro* models, meeting regulatory demands, and protecting human health, for an ethical and sustainable future.

Presentation: Oral



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Responsibility: Ethical considerations and scientific soundness in DIT testing

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The concept of the 4th R of “Responsibility” has increasingly been recognized as an essential addition to the traditional 3Rs (Replacement, Reduction, Refinement), reflecting the evolving ethical and scientific landscape of modern research. Unique challenges within developmental immunotoxicity testing (DIT) emphasize the need to consider the 4th R in the field. This presentation will highlight the critical role of responsibility in ensuring both ethical integrity and scientific validity within DIT research, reflecting on how this principle influences researcher obligations, study design, and public transparency.

The inclusion of “Responsibility” expands upon these foundational principles by embedding additional ethical and scientific dimensions into DIT strategies. Ethically, responsibility encourages transparency in experimental designs, promotes rigorous welfare assessments, and emphasizes proactive adoption of non-animal New Approach Methods (NAMs), including microphysiological systems, *in vitro* assays and computational tools. Scientific responsibility underlines the necessity of robust, reproducible, and predictive results through Good Cell Culture Practices (GCCP), validated assays, optimized study designs, and advanced technologies including AI-driven analyses and automated monitoring systems.

Recent advances in DIT testing exemplify the application of responsibility, including the development of NAMs, standardization of sensitive assays like cytokine production measurements, flow cytometric analyses, and cross-sector collaboration among academia, industry, and regulatory agencies to refine strategies and promote reproducible practices. Additionally, adherence to comprehensive reporting frameworks such as the ARRIVE (Animal Research: Reporting of *In vivo* Experiments) and RIVER (Reporting *In vitro* Experiments Responsibly) guidelines ensures transparency and reproducibility, reinforcing public trust and accountability.

In conclusion, integrating “Responsibility” into the 3Rs framework significantly enhances the ethical integrity and scientific rigor of DIT. This multidimensional approach not only mitigates ethical concerns but also substantially improves the validity, reliability, and translational relevance of immunotoxicity assessments, ultimately safeguarding public health and fostering greater societal trust in scientific research.

Presentation: Oral

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Underlying social barriers to change – Mapping social barriers to the transition to animal free science and safety assessment in order to overcome them

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As the practice of animal testing is coming under increasing scrutiny for its issues surrounding reliability, reproducibility and translation to human relevance, safety assessment systems around the world are transitioning in different ways in order to accommodate the use of new approach methodologies (NAMs) without the use of animals. The increasing demands of an ever-growing number of chemicals and pharmaceuticals which need to be assessed for their safety is facing regulatory systems which are increasingly under strain by a lack of human and financial resources. Meanwhile the use of NAMs requires a new way of interpreting data and making regulatory decisions, a variety of specializations, and a reorganization of legal-political arrangements; in short, a non-animal NAMs safety assessment requires a transformation of the current safety assessment regime.

Sociotechnical transition and transformation scholars have pointed out the importance of addressing the underlying causes underpinning transitions. Underlying causes and the social barriers which arise from them are therefore a key part of understanding the transition to animal-free science and safety assessment and form a crucial avenue for researchers and policymakers to understand how to accelerate the transition.

Science and technology studies (STS) have highlighted just how much the worldview, values and perspectives of scientists play a role in the creation of scientific data. Social and cultural factors are key determinants of how scientific knowledge is created and should therefore be taken into account in risk assessment systems.

The task of mapping the social barriers to accelerating animal-free science is still in its infancy; however, the research that has been done highlights biases for animal models and against NAMs, the conservatism of regulators to change, the institutional practices and discourses which discourage experimentation and the adoption of new data requirements.

This presentation will draw on fieldwork, interviews, and literature reviews on risk assessment in the EU and USA in the chemical and pharmaceutical sectors to delve deeper into the origins of certain re-occurring social barriers which hinder the transition. It will combine insights from STS, risk assessment studies and apply them to the case of animal testing in risk assessment. It will give a framework which better explains why the same social barriers can be seen in multiple sectors and across the world in both the Global South and Global North, as well as give initial recommendations on how to overcome them.

Presentation: Oral



598

Modeling human diversity: Sex differences in *in vitro* brain development

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Historically, human diversity has been underreported in biomedical research, limiting the applicability of research findings across different populations. This gap is particularly evident in *in vitro* models, where factors such as sex, ethnicity, and genetic diversity are often overlooked. Recent studies emphasize how integrating these variables can boost physiological relevance and better capture the range of human responses to drugs and toxicants.

In line with the session's aim to discuss human diversity in *in vitro* models, we investigated the role of biological sex in early brain development using a human brain microphysiological system (bMPS). Specifically, we examined how testosterone-induced masculinization influences neurodevelopmental processes *in vitro*. For this, induced pluripotent stem cells (iPSC) from male and female donors were differentiated into three-dimensional brain spheres consisting of multiple neuronal subtypes, astrocytes, and oligodendrocytes. During a sensitive differentiation window, these bMPS were exposed to testosterone and assessed for changes in cell composition and gene expression. Testosterone exposure resulted in a larger sphere size, mirroring reported sex differences in brain volume. Utilizing bulk RNA-sequencing, we observed transcriptional changes related to brain patterning and potential links to neurodevelopmental disorders.

Our findings underscore the value of incorporating sex hormones to model early brain development more accurately in human-based systems. This approach serves as a critical step towards more diverse and representative *in vitro* models – enhancing hazard identification, providing deeper mechanistic insights, and ultimately reducing biases and reliance on animal testing.

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Presentation: Oral

601

Innovative approaches for characterizing the health and environmental risk of chemicals using mechanistic NAMs

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A main challenge for the incorporation of NAMs in regulatory chemical risk assessments is the difficulty for applying the current paradigm, and in particular, the idea of a hazard characterization focused on setting a “threshold” level, at which no effects are expected, such as the daily acceptable or tolerable doses. Any challenge also represents a new opportunity; and moving towards animal-free approaches offers the opportunity for designing more mechanistic-based informative assessments. We revisit the regulatory process from a Public Health and Environmental perspective, to answer if the current paradigm is still the best science-based approach for supporting relevant decisions in Q2 of the 21st century. Our conclusion is that setting a non-effect threshold level is just a legacy from the past, which was based on “observational” toxicology. Setting thresholds is not a regulatory requirement, in fact the decisions are based on the integration of different elements, including the uncertainty around the assessment and policy considerations on the level of risk or risk-benefit assumed to be acceptable in each particular context. In fact, a more informative risk characterization, e.g., identifying the risk drivers under different conditions, seems to be much more relevant for decision makers than the current binary (above or below the threshold) simplification.

Following this conclusion, a set of alternative approaches are discussed as proposals for presenting the main outcome, the hazard and risk characterizations, covering regulatory assessments under different sectors and legislative frames.

New tools, such as High Throughput Screening (HTS) bioactivity analysis, quantitative Adverse Outcome Pathways (qAOP), spatially explicit modelling, or exposure-led hypothesis driven Next Generation Risk Assessments (NGRA), provide the methodological basis for understanding the interaction of chemical exposure with biological structures at different levels of organization. These are informative-rich approaches, which, in combination with comprehensive hazard characterization tools, offer excellent capacities for supporting decision makers and facilitating the integration of different risk characterization lines from complementary assessments.

The examples provided in this presentation include a NGRA framework for identifying HTS-based vulnerability drivers for population subgroups with specific diseases, probabilistic approaches linking qAOP with measurable health indicators, population dynamics models for integrating the overall risk of different pesticides used at the same or subsequent timings in the same crop, landscape spatial explicit models as building blocks of system-based approaches, and the use of the One



Health concept for integrating the risks and benefits for human, plant, animal and environmental health in sustainability impact assessments.

Overall, these innovative hazard and risk characterization approaches avoid oversimplification into “YES/NO” responses and facilitate a comprehensive and transparent use of scientific knowledge and uncertainties when taking decisions for protecting human health and the environment.

Presentation: Oral

602

New approach methodologies (NAMs) as part of the solution for the simplification of EU chemical legislation (REACH)

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The European Commission has adopted new proposals that will cut red tape and simplify EU rules for citizens and business. In the recent Competitiveness Compass, the Commission set out its vision to make the EU's economy more prosperous and competitive, building on the recommendations of the Draghi report. To regain competitiveness and unleash growth, the EU will need to foster a favorable business environment and ensure that companies can thrive. Resulting from the Chemicals Strategy, multiple EU regulations are under revision with the goal to simplify the regulation of Europe chemicals legislation (such as REACH Q4 2025). In this context, the regulatory use of NAMs for better chemical regulation is regularly requested by different stakeholders, however, to this date with low success. With the Commission's recent incentive to launch the roadmap to phase out animal testing, the simplification of REACH appears as a key opportunity while maintaining protection standards for human health and the environment. This oral presentation will explore how NAMs can be utilized as a solution for the simplification of REACH by 1) providing an overview of the multiples stakeholders action points ranging from trade association to non-governmental organizations 2) confronting commonalities and differences 3) ranking practicality and potential impact.

Presentation: Oral

603

Paradigm shift in progress: Witnessing the worldwide adoption of animal-free safety science

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Safety science has advanced a great deal since the 1950s when the only tools for assessing safety were animal tests. Decades of investment in New Approach Methodologies (NAMs) and Next Generation Risk Assessment (NGRA) approaches have enabled a global paradigm shift as animal-free safety science is increasingly adopted for regulatory use.

We have learnt that combining NAM data with exposure information using computational approaches enables us to set, and assess against, more meaningful human protection goals. As a result, it has become clear that transitioning to NAMs and NGRA approaches can help us better protect people, support sustainable innovation and phase out regulatory animal testing.

So where are we today? After two decades of rapid progress, we find ourselves at a tipping point as we collectively transition from early adoption to widespread regulatory use of animal-free safety science. So, what have we learnt and where do we go from here?

This talk seeks to offer a global perspective as we witness a regulatory paradigm shift towards the adoption of animal-free safety science and reflect upon how we can overcome remaining challenges together.

Presentation: Oral



604

HepaRG – An advanced hepatocellular system for genotoxicity assessment

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Genotoxicity New Approach Methodologies (NAMs) are increasingly becoming an important component of *in-vitro*-only genotoxicity assessment strategies. Not only do NAMs provide mode of action information important for distinguishing between direct and indirect genotoxicity, they do so while adhering to 3R principles (i.e., replace, reduce and refine). The metabolically competent human hepatic cell line HepaRG has successfully been implemented in a number of NAM approaches in recent years and provides a more accurate representation of human metabolism than traditional exogenous metabolism sources such as rat liver S9. The work presented here will include research and case studies demonstrating the benefits of this cell line for genotoxicity risk assessment, primarily using the high-throughput *in vitro* flow cytometric micronucleus assay. The first example will include the use of HepaRG for potency ranking of a large number of botanical contaminants of regulatory concern, which require metabolic activation to exert their genotoxic potential. The second example will demonstrate, via testing of a diverse set of 28 compounds, that HepaRG cells have similar predictive capacity when compared to TK6 cells, which are frequently used in regulatory approved OECD-guideline compliant *in vitro* micronucleus assays. The 28 compounds examined include non-genotoxic and genotoxic compounds with both direct and indirect mechanisms of action. Notably, in this dataset, HepaRG cells were less susceptible to false or irrelevant responses (i.e., *in vitro* positive, but *in vivo* negative) due to oxidative stress mediated indirect genotoxicity. In the final example, proof of principle research will be presented demonstrating the compatibility of this cell line to evaluate mutational signatures via error corrected sequencing technologies, an endpoint that thus far has primarily been evaluated using *in vivo* approaches. Taken together, the presented results are thought to demonstrate that the HepaRG cell line is a relevant replacement of animal studies that can support assessment of human genotoxicity risk in a regulatory context.

Presentation: Oral

607

Advancing defined approaches for developmental neurotoxicity using the integrated testing and assessment framework template

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The significant increase in neurodevelopmental disorders among children in recent decades has raised concern about the potential impact of exposures to environmental chemicals. The use of the *in vivo* developmental neurotoxicity (DNT) test guideline has been limited due to its resource intensive nature in terms of cost, time and number of animals used. Through a significant collaborative effort by various stakeholders, a battery of 17 *in vitro* assays, the DNT IVB (*in vitro* testing battery) was developed to enhance DNT assessment. The Organisation for Economic Co-operation and Development (OECD) released a crucial guidance document “Initial Recommendations on Evaluation of Data from the DNT IVB” providing essential insights into the implementation and interpretation of the DNT IVB data. Moreover, integrated approaches to testing and assessment (IATA) case studies were developed for different problem formulations including hazard identification and characterization, screening and prioritization, and waiving of DNT *in vivo* studies. However, these documents lack a standardized framework and further interpretative guidance for decision making (e.g., how to make fixed interpretation procedures of the DNT IVB data).

The IATA framework template was therefore applied to identify modules within the DNT IATA workflow that can be standardized and where flexibility needs to be maintained. Using two IATA case studies for i) hazard identification and ii) screening and prioritization this will be exemplified. The template starts with the problem formulation (purpose) as it will impact which data modules to integrate with the DNT IVB and the interpretation of the results. For example, IATAs for hazard identification and characterization often include systematic reviews of all available data with standardized bias assessment to determine level of confidence in the data. This is also needed in the vast majority of chemical legislations for chemical risk assessment. If the purpose is screening and prioritization a lower level of confidence is often acceptable why time-consuming systematic reviews may be optional. Integration of various toxicology studies and high throughput screening data from e.g., the Integrated Chemical Environment (ICE) and EPAs CompTox program could instead be considered. Guidance on standardized extraction and interpretation of such data would then be desired. Similar guidance for physiologically based toxicokinetic (PBTK) modelling and *in vitro* to *in vivo* extrapolation (IVIVE) has been proposed with specific consideration of the barriers (placenta and blood brain), brain as the target and developmental stages of the fetus or infant.

Working towards defined approaches for DNT will need further standardization for data analysis (e.g., pipeline methodology cut off, quality controls process, point of departure standardization), data integration (e.g.,



endpoints included, inclusion of cytotoxicity), interpretation (e.g., weight of assays, link to an adverse outcome pathway) and uncertainty assessment. The use of the IATA framework template will enhance the regulatory application of the DNT IVB as it provides a consensus-based, transparent and standardized testing strategy.

Presentation: Oral

617

Brazilian regulatory perspective on the substitution and removal of animal testing for veterinary vaccines

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In Brazil, the Ministry of Agriculture is the responsible for the veterinary vaccines regulations which serve for the registration, quality control and release of these key products. Many of those regulations include animal testing to secure the quality, safety and potency of the vaccines. Thanks to the investment made in the vaccine production to introduce strict controls over starting materials and development of Good Manufacturing Practice, Quality Assurance and Control and Pharmacovigilance systems, a proper environment is now in place in Brazil where animal-based tests could finally be substituted or removed. The Ministry of Agriculture has successfully collaborated with key stakeholders to allow the waiver of TABST and LABST. Dr Leandro Barbieri de Carvalho, coordinator at the veterinary products registration department, will present the work done by the Ministry to waive TABST and LABST and the other potential areas where animal testing can be substitute, the challenges that needs to be overcome in Brazil to successfully introduce further changes and how regional collaboration might further improve such transformation.

Presentation: Oral

621

Ethical and effective surgical skills acquisition: An overview of non-animal methods and clinical learning opportunities

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This presentation will explore the trajectory of clinical and surgical skills acquisition for students and trainees, and will provide an overview, with examples, of the range of non-animal tools and patient-based approaches that can optimize this process. It will cover medical and veterinary medical education, advanced surgery training, and continuing professional development for practitioners. The potential of these humane methods (“alternatives”) to replace harmful animal use – defined by InterNICHE to mean dissection of purpose-killed animals, animal experimentation, and other instrumental animal use – is also addressed.

In pre-clinical anatomy, pathology, physiology and pharmacology, effective knowledge acquisition is necessary to provide a solid foundation for the rest of a degree. In the clinical years, skills acquisition refers to a range of important and inter-related skills including technical competence and confidence; critical thinking and clinical reasoning, including use of knowledge; emotional literacy, including compassion and self-awareness; and ethical literacy, including a sense of responsibility.

For internal medicine and for clinical and surgical skills, humane methods for skills acquisition include the following: digital video, animations and apps; virtual and augmented reality (VR/AR); models, mannekins and simulators of diverse functionality and various levels of fidelity; simulated clinical scenarios, which might employ software linked to models; ethically sourced animal specimens, including those with added perfusion and pulsation; and supervised clinical learning opportunities with patients during scheduled rotations and placements, such as in teaching hospitals or via a distributed learning model.

These methods are typically employed in practical classes individually and in combination, and at appropriate points within the degree, according to the skill sets being addressed. Many also apply to advanced surgery training during specialization after graduation, and to continuing professional development. Critical reflections on the learning process and the curriculum, awareness of new technologies and creative opportunities, and active engagement with the ethical issues that education and training present, can allow for considered choices and skillful application of such methods. In turn, the humane innovations employed can often facilitate the process of meeting standard teaching objectives, and they can provide opportunities for additional objectives to be met. This can lift the quality of clinical and surgical skills education and training, and thereby improve patient safety and clinical outcomes.

Presentation: Oral



623

Organoid intelligence for developmental neurotoxicity testing: Modeling chronic domoic acid exposure

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Organoid Intelligence (OI) is emerging as an approach to studying neurodevelopment and neurotoxicity by integrating human brain organoids with advanced sensors and artificial intelligence (AI). This multi-modal *in vitro* and *in silico* platform enables the modeling of human functions relevant to learning and memory, offering an alternative to traditional animal-based cognitive studies. Classical *in vitro* models are valuable for studying acute neurotoxic effects but are less suited for assessing chronic exposures, as they often lack the prolonged survival, neuronal maturation, network development, and complex cell-cell interactions necessary to capture long-term effects. Traditionally, developmental neurotoxicity (DNT) testing has incorporated animal models; however, species differences can limit their ability to fully predict human-specific outcomes and many chemicals remain untested due to high cost and complex nature of those tests (DNT testing is not mandatory unless it is a pesticide or there is a trigger). Studying domoic acid (DA) is crucial due to its increasing prevalence as a marine neurotoxin linked to harmful algal blooms, which are becoming more frequent with climate change. While acute high-dose DA exposure is well-documented for causing seizures and cognitive impairments, the effects of chronic low-dose exposure remain poorly understood. In this study, we present human induced pluripotent stem cell (iPSC)-derived brain organoids (brain MPS) as an advanced platform for developmental neurotoxicity (DNT) testing on the example of DA. We exposed maturing organoids to 0.10 μM , 0.32 μM , and 3.16 μM (DA) and evaluated the impacts on key events of neural development as outlined in OECD guidance document on DNT *in vitro* testing battery. We have not observed any changes in proliferation, neuronal differentiation and maturation, and glia migration. However, electrophysiological recordings of spontaneous and evoked network activity, synaptic plasticity and analyses of oligodendrocyte maturation markers revealed alterations. Exposure to 0.1 μM and 3.16 μM DA increased spontaneous bursting ($P \leq 0.01$, $P \leq 0.0001$). The 3.16 μM group also showed more spike-sorted neurons ($P \leq 0.01$). Despite increased spontaneous activity, evoked cortical excitability was reduced in both DA groups ($P \leq 0.05$ and $P \leq 0.005$). Short-term plasticity was also diminished in the 3.16 μM group ($P \leq 0.05$). DA-exposed organoids exhibited impaired oligodendrocyte maturation, reflected by decreased expression of myelination markers and delayed oligodendrocyte development compared to controls.

Using domoic acid (DA) as a model compound, we are further advancing our Organoid Intelligence (OI) platform to develop learning-on-chip systems through open- and closed-loop experiments with aim to bring *in vitro* models closer to *in vivo* functionality, capturing dynamic processes in neuronal circuits. This approach not only enhances our ability to study neurotoxic effects on cognitive function but also establishes a foundation for more predictive, human-relevant models that can ultimately replace traditional animal-based studies in neuroscience and toxicology.

Presentation: Oral

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How industry and regulatory innovation and collaboration can promote the transition from *in vivo* to *in vitro* potency testing for human vaccines

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Vaccines Industry has been rolling out a strategy to phase out animal use for quality control testing, aiming to implement more reliable and scientifically relevant assays and address ethical concerns. Although developing and implementing *in vitro* assays on new products which are well characterized is commonly accepted, it is more challenging to introduce non animal alternatives on established vaccines which have shown consistent efficacy and safety over time through *in vivo* testing but are not as well characterized.

With the aim of facilitating regulatory acceptance of these *in vitro* alternatives, a general chapter (5.2.14) was introduced in the European Pharmacopoeia in 2018, providing guidance on how to proceed in order to substitute *in vivo* methods by *in vitro* alternatives for established vaccines. We will present the main considerations developed in this chapter with a particular focus on potency tests, emphasizing the complexity of comparing *in vivo* to *in vitro* assays given their different read outs.

In parallel, developing and validating alternative, non-animal testing approaches for both human and veterinary vaccines as well as contributing to their regulatory acceptance for vaccine quality control testing was the main goal of a public-private consortium called VAC2VAC (standing for "Vaccine batch to Vaccine batch comparison by consistency approach"), which brought together scientists from the pharmaceutical industry along with academia and regulators from 2016 to 2022. We will share some key outcomes of the VAC2VAC consortium and in particular the case study for human DTaP (Diphtheria, Tetanus and acellular pertussis) vaccines, one of the vaccines which account for the most animal use in quality control today, for which alternative immunoassays were developed, and critical reagents were made broadly available for potency testing. The next steps for manufacturers are to further develop, optimize and validate these assays for specific products.

This work was funded by Sanofi.

Emmanuelle Coppens is a Sanofi employee and may hold shares and/or stock options in the company.

Presentation: Oral



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Towards phasing out acute toxicity *in vivo* testing in EU chemical safety assessments

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To this day, in the European Union (EU) the potential acute toxicity of chemicals is still mostly assessed based on results from *in vivo* testing in rodents. In July 2023, the European Commission (EC) announced the preparation of a “Roadmap towards phasing out animal testing in chemical safety assessments”, expected to be published early 2026. In the context of the roadmap work, the replacement of *in vivo* testing with *in silico* predictions as the default methodology for acute oral toxicity (AOT) assessment under EU chemicals legislation has been identified as having great potential in the short term. Here, we report on the outcome of this work performed by an international expert group towards this target.

First, we present results of an analysis of the legal frameworks within the scope of the EC roadmap with respect to the legal requirements/regulatory needs as well as to a possible need for updating legal or guidance texts in case *in silico* methods were to become the new standard methodology for assessing AOT.

Next, possible benchmarks for acceptable predictivity of *in silico* models AOT are discussed and progress with the curation of a large AOT reference data set and the subsequent performance assessment of the CATMoS *in silico* tool (doi:10.1289/ehp8495) is reported as a pilot for the validation of further *in silico* tools in the future.

In addition, the presentation will provide an outlook on mid- to long-term activities envisaged under the roadmap, directed towards investigating potentially acceptable conditions for waiving AOT testing, towards complementing *in silico* methods with suitable *in vitro* test designs for out-of-domain chemicals and for developing animal-free strategies for the dermal and inhalation exposure routes as well.

Presentation: Oral

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The human exposome project: A moonshot initiative to transform environmental health

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The Human Exposome, encompassing all physical, chemical, biological, and psychosocial influences that impact biology, requires comprehensive characterization to translate its potential into medicine and public health. It complements the Human Genome Project by addressing the exposure side of disease. Achieving this goal demands not only scientific innovation but also a coordinated global effort involving governments, international organizations, scientists, funders, the technology sector, and the public – a “moonshot” approach to translating the exposome from concept to utility.

The Human Exposome Project is an ambitious scientific endeavor, inspired by the transformative Human Genome Project, aiming to systematically catalog environmental exposures and their impacts on health. Approximately 70-90% of chronic diseases have environmental rather than genetic roots, highlighting the need to map and understand the exposome. Using advanced sensors, AI, precision analytics, and predictive biological tools, the project will revolutionize public health by enabling precise interventions based on robust, real-time data instead of retrospective epidemiological analyses.

As a novel concept, we propose Exposome Intelligence (EI), an AI-driven approach integrating high-throughput chemical screening, microfluidic technologies, and machine learning to assess health effects of complex exposures. EI will leverage biomonitoring technologies and biomedical knowledge graphs to map exposure-disease relationships at an unprecedented scale. This will enable more efficient, data-driven, human-relevant prediction and mitigation of environmental risks.

The adoption of EI and AI-powered exposomics will also reduce reliance on animal testing in toxicology and public health research. Instead of extrapolating from animal models, which often fail to translate to human outcomes, the Human Exposome Project will employ human-relevant *in vitro* models such as organoids, human-on-a-chip systems, and advanced computational methods. These next-generation approaches align with the principles of the 3Rs (Replacement, Reduction, and Refinement), improving both ethical standards and scientific precision in environmental health research.

Significant political, financial, and infrastructure investments will be necessary to realize the full potential of exposomics, ensuring benefits in public health, longevity, economic productivity, and social equity. Based on insights from the Exposome Moonshot Forum, we propose a Roadmap toward the Human Exposome Project, outlining an actionable plan for exposome characterization and utilization. This plan includes implementation frameworks, governance structures, and collaborative partnerships across academia, industry, and government, emphasizing ethical data integration and AI-driven analytics. Ultimately, this initiative will redefine public health strategies and advance regulatory science by promoting human-relevant, non-animal methodologies aligned with the 3Rs.

Presentation: Oral



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Fostering ethical leadership through humane learning: Building critical thinking for innovation in medicine and biomedical science

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The tools and approaches employed in biology, medical and veterinary medical degrees have increasingly moved from conventional animal-based methods to humane and innovative ones. Student awareness and pressure, technological innovation and a critical focus on teaching objectives have brought progressive changes to many curricula. This presentation will explore the range of alternatives now widely in use, and how humane education supports the acquisition of technical competency, critical thinking skills, and ethical and emotional literacy. These skills can encourage a proactive, questioning mindset, and empower students and graduates to champion innovation not only in education but in the practice of medicine and biomedical science. The differences between education on the one hand, and medicine and science on the other, will be addressed, and the commonalities and pathways between humane education and humane careers will be highlighted.

Presentation: Oral

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Mind the (regulatory) gap: Accelerating acceptance of complex *in vitro* models through adverse outcome pathways

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The global demand to evaluate the toxicological risks of over 350,000 registered chemicals and mixtures exceeds both the capacity and predictive power of conventional animal-based testing methods. Despite significant advancements in complex *in vitro* models (CIVMs), which demonstrate robust validation and high fidelity with vastly improved predictive capacity over animal models, their integration into regulatory risk assessment guidelines remains almost non-existent due to persistent challenges with standardization, perceived uncertainties, and the lack of clearly defined evaluation frameworks and acceptance criteria. Adverse Outcome Pathways (AOPs) – structured conceptual frameworks capturing mechanistic sequences of biological events that causally link molecular initiating events through intermediate key events to adverse outcomes – offer a powerful solution by providing a pragmatic approach to guide and anchor the development, validation, and regulatory acceptance of CIVMs. During this critical transition toward regulatory acceptance, explicitly anchoring CIVMs to specific, well-characterized AOPs facilitates clarity in their intended contexts-of-use, directly addressing uncertainties regarding mechanistic relevance and predictive utility. To illustrate the feasibility of this AOP-guided approach, we highlight our recently developed human 3D-bioprinted liver tissue explicitly anchored to the well-characterized AOP-38 (liver fibrosis). We further demonstrate how extensively validated liver spheroids and organ-on-chip models from the literature, although not originally anchored to specific AOPs, can similarly be mapped to mechanistic pathways such as AOP-38, enabling their strategic use along a biological continuum of complexity. Collectively, these models exemplify how targeted alignment with established AOPs ensures biological fidelity, reproducibility, specificity, and sensitivity, providing a practical foundation for a structured integration framework that emphasizes clearly defined contexts-of-use, standardized performance criteria, robust validation against human-relevant data, transparent reporting practices, and explicit acceptance criteria to pragmatically bridge the gap between advanced CIVMs and their routine regulatory use.

Presentation: Oral



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The regulatory “R” in the 5Rs of developmental immunotoxicity testing: A framework for regulatory integration of new approach methodologies

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Traditionally, Developmental Immunotoxicity (DIT) assessments have relied on animal-based tests but growing scientific and ethical concerns have driven a shift toward human-relevant testing strategies. The Regulatory “R” plays a crucial role in facilitating the adoption of New Approach Methodologies (NAMs), including *in vitro* systems, *in silico* modeling, and computational toxicology approaches, that offer viable alternatives to traditional animal-based DIT studies.

Despite progress, several barriers hinder the regulatory adoption of NAMs. A lack of standardized and validated methodologies remains a primary challenge. To advance regulatory acceptance of NAMs in DIT testing, several key pathways must be pursued. The OECD’s AOP framework provides a structured approach for linking molecular and cellular events to adverse immune system outcomes, enabling the development of regulatory-relevant biomarkers. High-throughput transcriptomics, metabolomics, and epigenetics are enhancing the mechanistic understanding of immunotoxicity, allowing for more precise hazard identification.

Further progress requires continued investment in the validation of regulatory-ready NAMs. Advanced human-relevant models, such as immune organoids and microphysiological systems, are demonstrating significant potential for evaluating immune development and function. *In silico* and machine learning-based approaches are also gaining traction as predictive tools for immunotoxicity risk assessment. To fully leverage these innovations, global regulatory alignment must be strengthened, encouraging collaboration between academia, industry, and policymakers to drive consensus on alternative DIT testing strategies. Open science initiatives, including the NIEHS/NICEATM Integrated Chemical Environment, are essential for ensuring regulatory access to transparent, high-quality NAM datasets.

The Regulatory “R” is critical to achieving a transition from traditional DIT testing to predictive, human-relevant, and ethical methodologies. By fostering collaboration among scientists, regulators, and policymakers, NAM-based approaches can improve chemical risk assessments and enhance public health protection.

Presentation: Oral

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Brazilian stakeholders toward 3Rs implementation

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The principles of the 3Rs – Reduction, Refinement, and Replacement of animal use in research and experimentation – were first introduced in 1959 by scientists William Russell and Rex Burch in their seminal work, *The Principles of Humane Experimental Technique*. Their vision was to establish a framework that not only minimized animal suffering but also encouraged the development of alternative methods without compromising the integrity and validity of scientific research. Over the decades, the 3Rs have evolved into a cornerstone of ethical and sustainable scientific practices worldwide.

In Brazil, the adoption and implementation of the 3Rs involve a multifaceted ecosystem of stakeholders, including regulatory agencies, academic institutions, and private sector initiatives, all working collaboratively to advance alternatives to animal use. At the heart of this effort is the National Council for the Control of Animal Experimentation (CONCEA), which oversees the regulation and ethical use of animals in research. CONCEA establishes guidelines that prioritize the reduction of animal numbers, the refinement of experimental procedures to enhance welfare, and the replacement of animals with alternative methods wherever feasible.

Supporting this regulatory framework are organizations such as the National Network of Alternative Methods (RENAMA) and Brazilian Center for the Validation of Alternative Methods (BraCVAM), which play pivotal roles in developing, validating, and disseminating innovative methodologies. RENAMA, established in 2012 under the Ministry of Science, Technology, and Innovation (MCTI), provides training for researchers and industry professionals, facilitating the adoption of these methods through courses, workshops, and the distribution of best practices. The network operates collaboratively, linking research institutions, universities, government agencies, and the private sector to foster cooperation and knowledge sharing. Additionally, RENAMA contributes to the formulation of public policies and regulations that promote the use of alternative methods in accordance with international guidelines. Regional initiatives like PREMASUL further bolster these efforts by offering training programs and fostering research into alternative approaches.

The integration of these methods into industry practices is facilitated by regulatory bodies such as the National Health Surveillance Agency (ANVISA), particularly in the cosmetics, pharmaceutical, and chemical sectors. These agencies ensure that alternative methods are not only recognized but also safely and effectively implemented, aligning with global trends toward ethical science.

The progress of the 3Rs in Brazil reflects a collective commitment among diverse stakeholders to advance scientific innovation while upholding ethical standards. This collaborative approach underscores the country’s dedication to fostering a more humane and sustainable future for research and experimentation.

Presentation: Oral



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From concept to consumer: Bridging global standards and regional practices for safety by design

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The Safety by Design (SbD) approach represents a proactive framework that integrates critical safety attributes – such as sensitizers, mutagenic impurities, nitrosamines, and extractables and leachables – into the Quality by Design (QbD) principles outlined by ICH. By embedding safety at the earliest stages of pharmaceutical development, SbD enhances risk assessment strategies, reduces uncertainties, and ensures the creation of inherently safer products. This framework provides a robust methodology for minimizing risks throughout the entire drug development lifecycle. The SbD approach emphasizes the early identification of hazards, promoting a shift from traditional post-event remediation to proactive risk mitigation. Key Critical Process Parameters (CPPs), such as moisture, light exposure, pH, and temperature control, are critical factors influencing impurity formation and drug stability. By addressing these parameters from the outset, SbD helps pharmaceutical developers avoid costly safety failures and ensures that drugs are safe and stable from concept to consumer. Integrating SbD principles into the development process not only improves product quality but also fosters sustainability by aligning with global safety standards. Furthermore, SbD supports the application of advanced technologies, including computational modeling and predictive analytics, which enable earlier hazard assessments with fewer resource-intensive testing requirements. This innovative use of technology enhances efficiency and accelerates the development process while maintaining rigorous safety standards. A critical aspect of SbD is its alignment with evolving global regulatory frameworks, ensuring that companies can meet both current and future regulatory expectations. The growing demand for compliance with international safety standards is especially relevant in regions such as Latin America, where adherence to global frameworks can significantly enhance market access and consumer trust. By fostering collaboration across industry, academia, and regulatory bodies, SbD drives the continuous exchange of knowledge, establishes best practices, and promotes the development of standardized safety protocols. This collaborative approach ensures that pharmaceutical companies are well-equipped to meet global safety standards while addressing regional practices and challenges. SbD is not merely a strategy but a comprehensive philosophy that underpins every phase of pharmaceutical development. Through proactive risk identification and continuous improvement, SbD enables the industry to create safer, more sustainable products, ultimately bridging the gap between global standards and regional practices. This presentation will explore how the SbD framework can be effectively integrated into the development process, providing attendees with actionable strategies to enhance product safety, regulatory compliance, and operational efficiency.

Presentation: Oral

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Perspectives on the implementation of new approach methodologies (NAMs) in Latin America: Findings from a survey conducted during the 5th COLAMA

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The application of New Approach Methodologies (NAMs) in chemical risk assessment models across Latin America is a key step in the regulatory science modernization process, as mandated by the 3Rs principles. The shift from traditional *in vivo* models to mechanistically informed alternative approaches is required for the attainment of a more ethical, efficient, and scientifically sound toxicological assessment paradigm. To investigate perceptions, obstacles, and strategies for NAMs adoption in the region, a systematic survey was conducted during the 5th Latin American Congress on Alternative Methods to Animal Testing (5th COLAMA), a key platform for scientific and regulatory discussions.

To analyze perceptions, challenges, and strategies for NAMs adoption in the region, a structured survey was conducted during 5th COLAMA. The survey gathered responses from professionals across various sectors, with academia representing the majority, followed by regulatory agencies, industry, and NGOs. Respondents from Colombia, Brazil, Mexico, Spain, and Chile provided a diverse perspective on the current state and outlook of NAMs adoption.

Based on a Likert scale between 1 and 5 (1 = strongly disagree; 5 = strongly agree), the main limitations identified were the insufficient governmental and regulatory support (4.39) and low investment in R&D (4.18). These limitations hinder the integration of NAMs into national and regional toxicological risk assessment models. Moreover, the participants highlighted the dispersed character of activities in Latin American nations, stressing the imperative necessity for regulatory coordination, trans-boundary cooperation, and the establishment of shared scientific and technological infrastructure. In the absence of concerted action, isolated progress will be prone to be compartmentalized, thereby restricting its influence on policy change and industrial practice.

Qualitative analysis of open-ended answers also underscores both advancement and structural or cultural barriers. Although institutional dedication and scientific curiosity are mounting, difficulties persist, including limited dissemination of scientific proof supporting NAMs, institutional inertia in blocking changes to regulatory paradigms, and the complexity of introducing alternative approaches into dominant legal frameworks. Brazil has frequently been cited as a regional benchmark because of its advanced technological infrastructure, dedication to alternative methods, and innovation in the validation and application of NAMs.

There were high agreement scores (≥ 4.00) on Likert-scale ratings of strategy options to enhance NAMs accessibility and integration. The highest rated strategies were expanding knowledge dissemination (4.35), strong and standardized method validation (4.27), and developing alternative methodologies especially for specific regulatory and industrial contexts (4.30). These findings emphasize the need to fos-



ter multidisciplinary collaborations, strengthen science and regulatory training programs, and create policy frameworks to facilitate the broad use of NAMs with consideration for Latin America's unique regulatory conditions and economic constraints.

Presentation: Oral

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N-nitrosamine risk assessment in pharmaceuticals: A safety by design approach

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According to ICH M7, N-nitrosamines are part of the cohort of concern due to their high carcinogenic potency and posing significant risks in pharmaceutical products. Their unintended presence in medications, notably angiotensin II receptor antagonists (sartans), has triggered global regulatory interventions, underscoring the need for robust, proactive strategies to prevent their formation. Safety by Design (SbD) offers such a framework, enabling the pharmaceutical industry to integrate risk mitigation early in the drug development process. By incorporating SbD principles from the earliest stages of development, predictive risk assessments can be made even when detailed information about the active pharmaceutical ingredient (API), formulation, and packaging is limited. The application of SbD involves a thorough analysis of the API's chemical structure to identify potential reactive sites that may be susceptible to nitrosamine formation. Furthermore, the identification of solvents and reagents that could catalyze such reactions, as

well as the evaluation of formulation and packaging components. This proactive risk identification helps embed safety measures into the drug design early on, reducing the likelihood of unforeseen risks. Key predictive tools, such as the Maximum Theoretical Formation Calculation and Kinetic Modeling, are employed to estimate potential nitrosamine formation. The first approach calculates the upper-bound risk based on nitrite content and the molar mass of the nitrosamine, while the second simulates real-world conditions such as pH, temperature, and reaction time during manufacturing, providing a dynamic evaluation of nitrosamine formation throughout the production process. When significant risks are identified, SbD promotes the early development of analytical methods to detect and quantify nitrosamines during production. Mitigation strategies include selecting excipients with minimal nitrite content, optimizing synthetic routes to reduce nitrosamine formation, and incorporating nitrite scavengers to reduce the potential for nitrosamine synthesis. Furthermore, careful selection of packaging materials ensures the integrity and safety of the final product. The integration of these strategies enhances both the safety and quality of pharmaceutical products, aligning with regulatory guidelines and ensuring that patient exposure to nitrosamines remains within acceptable thresholds. In addition, the role of emerging technologies in enhancing the effectiveness of SbD cannot be overlooked. By leveraging these advanced tools, pharmaceutical manufacturers can make informed decisions at every stage of the development process, further reducing risks associated with nitrosamine contamination. Furthermore, successful implementation of SbD requires close collaboration across interdisciplinary teams, including product development, quality control, and regulatory affairs. This collaboration ensures that safety is prioritized at every stage of the product lifecycle. The transformative potential of SbD in nitrosamine risk assessment will be demonstrated through case studies that showcase its application from the early stages of pharmaceutical development. These real-world examples will illustrate how SbD can preemptively identify risks, implement mitigation strategies, and enhance the safety profile of medications.

Presentation: Oral



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Advancing drug safety: Predicting mutagenic impurities in the early phases of pharmaceutical development

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Mutagenicity refers to the ability of a substance to induce genetic mutations by altering DNA base sequences. In the pharmaceutical industry, such substances are classified as mutagenic impurities (MIs) and can arise from active pharmaceutical ingredient (API) synthesis pathways or degradation processes throughout the drug product lifecycle. According to the ICH M7 guideline, these impurities must be identified, categorized, and controlled to mitigate potential carcinogenic risks to patients. The Safety by Design (SbD) approach is a proactive framework that integrates critical safety attributes – including sensitizers, mutagenic impurities, aflatoxins, and allergens – into the Quality by Design (QbD) principles established by ICH Q8-Q11. By defining Critical Quality Attributes (CQAs) and Critical Process Parameters (CPPs), SbD enhances risk assessment strategies and reduces uncertainties from the early phases of drug development. Key CPPs include moisture, light exposure, pH, and temperature control, which can significantly influence impurity formation and drug stability. Since API synthesis routes and degradation profiles remain largely undefined in the early F1 stage of development, predictive methodologies play a crucial role in identifying potential MIs. A multi-tiered approach is employed, including: Data mining from peer-reviewed literature and patent databases; *in silico* modeling, leveraging complementary predictive systems such as Derek Nexus (rule-based expert system) and Sarah Nexus (statistical model employing self-organizing hypothesis networks); Regulatory databases, such as the Carcinogenic Potency Database (CPDB) and the Lhasa Carcinogenicity Database (LCDB), which provide long-term carcinogenicity data for thousands of chemicals. By incorporating these methodologies, pharmaceutical developers can preemptively assess the risk of mutagenic impurities, enabling informed selection of API manufacturers and synthesis routes that align with safety and quality requirements. Additionally, the integration of SbD principles ensures that analytical development strategies account for impurity-specific detection needs, optimizing sensitivity thresholds and quantification limits to guarantee drug safety throughout its entire lifecycle. The transformative potential of SbD in MIs risk assessment will be demonstrated through case studies that showcase its application from the earliest phases of pharmaceutical development.

Presentation: Oral

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Mixture effect across the continuum of environment-food-human exposure: One approach, a multitude of answers

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In vitro bioassays in combination with chemical profiling can close critical gaps in the mixture risk assessment of chemicals throughout the environment-food-human continuum.

In the EU-funded project PANORAMIX, we developed a test battery of 22 *in vitro* bioassays that broadly covered developmental neurotoxicity, thyroid hormone system disruption, reproductive toxicity and genotoxicity. The adverse outcome pathway concept helped us to identify bioassays indicative of adverse outcomes and served as a starting point for the selection of bioassays. We prepared 16 complex chemical mixtures that were pooled extracts of wastewater, surface water, drinking water, lean and fatty fish, cow's milk, human breast milk and human serum samples (adult, cord blood) from a range of countries with the aim of representing "average European chemical mixtures". With this work on enriched extracts across the environment-human continuum, we observed adverse effects representing a high diversity of biological endpoints. All groups of mode of action were affected except for genotoxicity and 81% of the assays were active with wastewater down to 38% with surface water. Only few endpoints were active in drinking and bottled water, whereas 55-60% of assays were active when dosed with the fish extracts, 40-50% with milk extracts and 50-60% with serum extracts. In wastewater, effluent wastewater, surface water, fish, cow's milk, human breast milk, and human serum we detected 341, 188, 158, 27, 42, 47 and 160 chemicals, respectively, which illustrates that wastewater is the most contaminated extract, followed by effluent wastewater and human serum, which contained comparable number of contaminants. Studies on effect-directed analysis are ongoing to pinpoint which chemicals are driving the mixture effects.

Using a quantified subset of these chemicals in water, food and human tissue, we performed component-based studies to investigate potential interactions between the chemicals. For all matrices we observed additivity of the chemicals at low realistic concentration levels. Moreover, these studies allowed us to illustrate that when mixing chemicals at levels at which they did not exert any detectable effect by themselves, a marked mixture effect was observed. Thus, chemicals which act additively can still represent a potential risk. This subset of 24 chemicals could explain less than 1-10% of the total activity measured, meaning that many more chemicals contribute to the mixture effect.



In conclusion, we showed how chemical mixtures are transferred from the environment to humans via food and water. These complex chemical mixtures in water, food and human samples led to adverse reproductive and neurotoxic effects, as assessed in a battery of *in vitro* bioassays and we observed marked mixture effects on reproductive and neurotoxicity occurring at low concentrations when single compounds had no detectable effect.

Presentation: Oral

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Progress on the development of *in vitro* methods for assessing respiratory sensitizers

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The adverse outcome pathway (AOP) effectively describes the process of skin sensitization; however, the immunological mechanisms underlying respiratory sensitization remain poorly understood. As substances of very high concern (SVHC) under the EU regulation (REACH), respiratory sensitizers present a significant challenge for hazard identification. Existing *in vitro* models, designed primarily to identify skin sensitizers, are unable to distinguish between skin and respiratory sensitizers. This limitation underscores the urgent need for physiologically relevant test systems that accurately represent the respiratory tract to identify chemical respiratory sensitizers.

This presentation aims to provide an overview of the current *in vitro* assays available to predict the respiratory sensitization potential of chemicals. To date, only two commercialized models – GardAir® and ALIsens® – are available and capable of predicting respiratory sensitization. These models will be discussed in detail, including their applications and identified limitations. Additionally, emerging assays in the early stages of development will be introduced.

Our findings indicate that advanced *in vitro* alveolar models show promise in differentiating respiratory sensitizers from skin sensitizers and non-sensitizers, offering a more refined approach to hazard assessment.

Presentation: Oral

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Advancing the application of new approach methodologies (NAMs) for systemic toxicity assessment of cosmetic ingredients: An example with UV filters

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A next generation risk assessment was carried out to evaluate the safety of benzophenone-4 (BP-4), a UV filter present at 5% in a body lotion, to compare a non-animal approach with a traditional safety assessment based on historical animal data. Exposure characterization indicated that BP-4 is poorly absorbed through the skin, poorly metabolized by the liver, a substrate of influx and efflux transporters, and excreted by the kidney. The resulting physiologically-based kinetic model predicted a upper bound (95th percentile) plasma C_{max} of 1.27 µM, and liver and kidney concentrations of 0.32 µM and 0.44 µM, respectively. To characterize bioactivity, *in silico* and *in vitro* new approach methodologies were used. Points of departure (PoDs) were derived from four bioactivity platforms, including *in vitro* pharmacological profiling, CALUX assays, high-throughput transcriptomics, and a cell stress panel. The lowest PoD was from a single gene expression change, and the highest PoD from phenotypic biomarkers using a primary renal cell model. By dividing the *in vitro* PoDs from these assays by the 95th percentile plasma C_{max} value, so-called bioactivity:exposure ratios (BERs) were calculated. The BERs ranged from 3.3 to 493, indicating that no biological activities would be observed at consumer-relevant exposures of BP-4, confirming the outcome of independent traditional safety assessments based on historical animal test data. This work demonstrates the usefulness of next generation risk assessment in addressing pressing relevant regulatory questions without using animals.

Presentation: Oral



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Stem cell-based methodology for *in vitro* teratogenicity assessment

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Advancements in *in vitro* toxicology have been marked by key regulatory milestones, particularly in Brazil. Notably, the Arouca Law and the creation of the National Council for Animal Experimentation Control (CONCEA) in 2008 regulate animal use in research and education. In 2023, Brazil introduced a law mandating alternative methods for cosmetic evaluation. More recently, in November 2024, Brazil enacted Law 15,022, which establishes the National Inventory of Chemical Substances and regulates the risk assessment and control of chemicals produced or imported into the country. In the field of developmental and reproductive toxicity (DART), the evaluation of potential toxic compounds still requires the advancement of New Approach Methodologies (NAMs).

This presentation will highlight the approach of the Laboratory of Education and Research in *In vitro* Toxicology at the Federal University of Goiás, Brazil, in developing Alternative Methods and New Approach Methodologies (NAMs) for evaluating teratogenicity. Our focus has been on the development of stem cell-based methods for screening teratogens, such as a platform using dental stromal cells as a human-based model. This includes stromal cells from apical papilla (SCAPs), as well as platforms based on induced pluripotent stem cells (iPSCs).

Presentation: Oral

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Public-private partnership in predictive toxicology education: Example of the Sorbonne University – MatTek academy initiative

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The successful replacement of animal testing in toxicology depends not only on scientific advances but equally on raising awareness among all stakeholders about the limitations of historical approaches, newly available and developing methods, regulatory evolutions, and societal challenges. Education plays a fundamental role in building an ecosystem that is open and prepared for the paradigm shift required by 21st century toxicology. As a historical pioneer in the development and validation of alternatives to animal methods, MatTek recognized this need early on and developed hands-on training programs for *in vitro* methods. Building on its experience, MatTek established in 2022 a strategic partnership with Sorbonne University, creating a specialized course unit entitled “Predictive Toxicology and Tissue Engineering” for Master’s degree students in Biotechnology.

The course covers the principles of toxicological assessment, challenges of alternative methods, their development, validation, and implementation to meet both regulatory requirements and scientific standards for product safety. Particular emphasis is placed on biotechnological contributions, especially tissue engineering and 3D models, addressing both ethical concerns and scientific expectations in the field. The approach effectively combines theoretical lectures delivered by academic and industry experts with hands-on laboratory experience using validated methods for skin, eye or respiratory irritation testing.

Each year, the program intensively trains forty Master’s students during a one-week period. The cohort consists of students, half in work-study arrangements with academic laboratories, government agencies, or industrial partners, while the remaining students are ready for their six-month final Master’s internships. These students are therefore already in a professional environment but are still at a stage of their career with the potential for a diversified trajectory. Feedback from the first alumni shows the diversity of their career paths, in startups, multinationals or government agencies. Without being toxicologists, they possess the keys that will enable them to challenge and even steer evaluation strategies towards non-animal methods. This educational partnership represents a significant step toward building the human capital necessary for the widespread implementation of new approach methodology (NAMs), demonstrating how industry-academic collaboration can effectively prepare future professionals to advance the 3Rs principles in toxicology.

Presentation: Oral



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Innovative *in vitro* approaches for respiratory sensitization testing: Advancing beyond animal models

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Inhalation toxicity assessment is a mandatory requirement for chemicals and products that have the potential to enter the respiratory system. Currently, only *in vivo*-based methods, which involve the sacrifice of hundreds of animals annually, are validated for this purpose. Beyond ethical concerns and their limited physiological relevance, these methods primarily rely on lethality as the main endpoint for classification, providing little mechanistic insight into chemically induced pulmonary injury. As a result, despite the European Union's REACH regulation classifying low-molecular-weight (LMW) respiratory sensitizers as Substances of Very High Concern (SVHC), no validated methods exist for their preclinical toxicity and risk assessment. This gap is partially due to the incomplete understanding of the Adverse Outcome Pathway (AOP) of respiratory sensitization, as traditional *in vivo* models fail to adequately capture key immune activation events and lung tissue responses. To address these limitations, we developed an *in vitro* platform for assessing the respiratory sensitization potential of chemicals using cell lines derived from the respiratory system. By employing bioengineering strategies, we integrated these cell types into a physiologically relevant 3D-tetraculture bronchial model cultured at the air-liquid interface (ALI), effectively replicating the phenotype and function of human airways. To evaluate the model's responsiveness, we exposed it to aerosols of known respiratory sensitizers and assessed key cellular and molecular responses. Our findings demonstrated that exposure to sensitizer aerosols led to increased production of IL-6 and IL-8, elevated reactive oxygen species (ROS) generation, and induction of epithelial apoptosis, indicating chemically induced tissue damage. Moreover, the model enabled the evaluation of dendritic cell activation through IL-8 upregulation in monocytic cells, reinforcing its suitability for studying key mechanistic events within the AOP of respiratory sensitization. These findings highlight the potential of this 3D-tetraculture system as a valuable New Approach Methodology (NAM) for investigating chemically induced respiratory sensitization, addressing a critical gap in regulatory toxicology. Additionally, exposure to aerosols under ALI conditions significantly enhanced the physiological relevance of the model, making it a promising tool for preclinical airway toxicity assessment and advancing the development of non-animal alternatives for inhalation hazard evaluation.

Presentation: Oral

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NAMASTOX – The web interface to guide safety assessment through ASPA

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To facilitate actual application of ASPA and to make ASPA sustainable beyond ASPIS we developed a web-based tool, which can be considered a dashboard. For this we encoded the ASPA workflow in NAMASTOX. NAMASTOX is an open-source software that can be installed in most operative systems and environments. It is a user-friendly application that enables the user to upload data, in a secured environment if needed, to apply ASPA for a given hazard or risk assessment. This may involve a hazard or risk assessment under a certain regulation but could also involve an in-house assessment within a company. Additionally, NAMASTOX produces an informative risk assessment report, which comprises the documented results and expert decisions. Furthermore, this web-based tool can also help guide the less-experienced user through the process by providing guidance and linking to tools, such as the OECD QSAR toolbox, and to data sources, such as the U.S. EPA Comp-Tox Chemicals Dashboard.

In this presentation we will demonstrate through concrete case studies how the dashboard-like structure of NAMASTOX graphically interconnects the different workflow steps. These case studies cover different industry sectors and thus different regulations. Besides demonstrating the applicability of ASPA, we will also present how key resources of the ASPIS projects, such as the NAM- and the substance databases, are utilized for advancing NGRA approaches.

Finally, we will present the implementation of a new add-on, which is aimed at providing interactive uncertainty management for NAMASTOX. This new feature set supports users with i) the collection of uncertainty characterization results at each single workflow step, ii) the review of the collected uncertainty characterization results from relevant upstream nodes at the decision nodes, and iii) the execution of uncertainty quantification and combination methods, once provided and validated.

Presentation: Oral



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Chaotic bioprinting of human tissue models

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Layered microstructures are fundamental to mammalian tissues, granting them essential biological functions. However, replicating these intricate architectures remains a significant challenge with existing biofabrication methods. Here, we present advancements in chaotic bioprinting, an extrusion-based technique that leverages deterministic chaotic advection to generate complex multilayered and multimaterial constructs with unprecedented resolution and efficiency.

In chaotic bioprinting, multiple fluid-phase materials (typically alginate-based) are coextruded through a printhead equipped with a Kenics static mixer (KSM), which consists of helicoidal elements that repeatedly split and reorganize the material streams, forming a continuous filament with well-defined internal microarchitectures. As this filament is extruded, it undergoes ionic crosslinking upon contact with a calcium solution, solidifying into a hydrogel structure. Additionally, for formulations containing photopolymerizable components, secondary crosslinking via light exposure further stabilizes the constructs.

We demonstrate the versatility of chaotic bioprinting in fabricating tissue-like constructs that recapitulate native hierarchical architectures. For instance, engineered muscle-like tissues exhibit enhanced myogenesis when co-cultured with fibroblasts, improving their physiological relevance. Additionally, the integration of fugitive inks enables the creation of prevascularized constructs with perfusable microchannels as small as 22 μm , approaching capillary-scale dimensions. Beyond musculoskeletal applications, chaotic bioprinting adapts to structure materials with challenging interfacial properties, such as oil-in-hydrogel emulsions, and facilitates the fabrication of microgels with distinct compartments for applications in cancer modeling and drug testing. Moreover, it enables the generation of constructs with continuous axial and radial architectural transitions, closely mimicking natural tissue organization.

Overall, chaotic bioprinting emerges as a powerful and adaptable biofabrication strategy, enabling the creation of structurally complex, prevascularized engineered tissues and functional *in vitro* models. Its simplicity, scalability, and high resolution highlight its strong potential for translational applications.

Presentation: Oral

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Regulatory acceptance of alternatives to animal testing for foods

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The Brazilian Health Regulatory Agency (Anvisa) performs risk assessments of substances added to food, including novel foods, novel food ingredients and food additives, before the product is put on the market based on information provided by interested parties. In the processes evaluated by the Food Risk Assessment department, toxicological tests are evaluated to prove the safety of substances presented in the food. Although alternatives methods are desired to substitute animals testing, currently there is no validated method that provides as much information as animal tests. Discussions on the New Generation Risk Assessment (NGRA) are increasing, with the emergence of different international networks with the aim of replacing traditional methodologies of food risk assessment. New Approach Methodologies (NAMS) places an important role in NGRA discussions. While NGRA is not established, ANVISA has put effort in defining stepwise approaches accepting alternative methods such as the use of the Threshold of Toxicological Concern (TTC) when performing food risk assessment. In this approach, animals testing is restricted to those cases where there is a higher risk involved, and that more information is needed to assert about safety.

Presentation: Oral



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Regulatory acceptance of novel approach methods in support of medicinal product development

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The European regulatory network has a long-standing commitment towards the application of the principles of Replacement, Reduction and Refinement (3Rs). This is driven both by the requirements of EU Directive 2010/63/EU, as well as by the crucial need for better tools to predict quality, safety and efficacy of new medicinal products. In this context, the leverage and regulatory acceptance of non-animal approaches, including human-based complex *in vitro* models, *in silico* tools, etc., in the regulatory non-clinical testing of human medicinal products is fostered. Qualification of non-animal approaches is seen as important and requires discussion on and definition of regulatory acceptance criteria (e.g., context of use, endpoints and reference compounds).

To this end, the 3Rs Working Party (3RsWP), set up as the official 3Rs hub for at the European Medicines Agency (EMA), initiated a broad set of activities dedicated to fostering the 3Rs in regulatory testing of human as well as veterinary medicinal products. These include qualification of non-animal approaches such as complex *in vitro* models for specific contexts of use. Particular attention is given to stakeholder interaction and the initiation of an international regulatory conversation in order to harmonize views and acceptance criteria.

This presentation will provide a regulatory view on the specific challenges and opportunities related to the regulatory acceptance of non-animal approaches for the testing of human medicinal products. Experience gained from past related regulatory discussions (e.g., ICH E14:S7B Q&A, ICH S1B(R1), ICH S5(R3)) will be discussed, both with respect to acceptance of (human based) *in vitro* systems for a pre-defined context of use as well as with regards to the regulatory acceptance of weight of evidence approaches.

Presentation: Oral

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Regulatory acceptance of alternatives to animal testing for medicines

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The Brazilian Health Regulatory Agency (Anvisa) was established in 1999 by Law No. 9.782/1999, with the responsibility of regulating, controlling, and overseeing products, services, and substances that may affect public health.

Although no regulatory framework impeded the implementation of alternative methodologies for animal testing in Brazil, Anvisa issued Resolution No. 35/2015 in 2015 to explicitly confirm the acceptance of methodologies aimed at replacing, reducing, or refining the use of animals in research activities.

In 2021, Anvisa adopted a more practical approach through the Brazilian Pharmacopoeia, incorporating alternative methods into the compendium to establish applicable criteria and to facilitate the education of the field regarding new methodologies. As a result, the Monocyte Activation Test (MAT) was included in the 7th edition of the Brazilian Pharmacopoeia, published in December 2024.

From December 2024 to March 2025, there was no increase in the number of post-marketing authorization requests related to pyrogen testing (transition from RPT to MAT). During the same period, no impact was observed on new drug applications due to the inclusion of MAT in the Brazilian Pharmacopoeia as the preferred method for pyrogen testing.

Conversely, companies have shown increased interest in proposing the use of *in vitro* tests to replace animal testing. However, most of these requests fail to provide comprehensive validation for the specific product and typically cannot demonstrate that the method is representative of the quality and safety criteria it intends to address.

This presentation will provide an overview of the regulatory acceptance of New Approach Methodologies (NAMs) for medicines in Brazil.

Presentation: Oral



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Non-animal approach for safety assessment of natural cosmetic ingredients from Amazonian biodiversity

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Natural ingredients obtained from Brazil's rich biodiversity offer a strategic approach that aligns with sustainability objectives for cosmetic companies. These natural components, commonly derived from seeds, flowers, and roots, often possess oily and grass properties, besides presenting a variability in their chemical composition. Frequently, there are many unpublished natural products for which there is no toxicological data available, impacting directly in the safety assessment of these raw materials.

New approach methodologies (NAMs) play a crucial role in non-animal strategies for the safety assessment of these substances. Integrated approaches, combining *in silico* and *in vitro* methods, are essential to evaluate the toxicological potential of botanical ingredients through a weight of evidence.

In silico models are very useful to predict the toxicological potential of natural products based on the molecular structure of each compound, besides guiding the need and choice of *in vitro* methodologies to complete the safety assessment.

In vitro testing is essential to assess the safety of these substances following non-animal approaches. However, evaluating oily raw materials in conventional cell cultures poses several technical and methodological hurdles, due to solubility and precipitation issues, for example. To overcome these challenges, researchers can adopt cell culture models that better mimic physiological conditions *in vivo*, such as three-dimensional (3D) cell culture models and microphysiological systems.

This presentation will explore a non-animal approach for assessing the safety of natural cosmetic ingredients derived from Amazonian biodiversity, emphasizing new approach methodologies available and developed to evaluate ingredients with limited historical use or scientific documentation. A case-study will be presented to illustrate the process adopted to perform the safety assessment of a botanical ingredient in a robust way, using new approach methodologies, to ensure the safety of our consumers.

Presentation: Oral

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The critical role of exposure data in fragrance safety assessment

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Exposure assessment is a cornerstone of fragrance material safety evaluation, providing essential data to support science-based decision-making. This session will outline RIFM's current exposure modeling framework, emphasizing its role in quantifying real-world use scenarios for fragrance ingredients. RIFM's exposure model leverages extensive data to determine aggregate exposure, incorporating consumer habits and product use patterns to refine risk assessments. The presentation will highlight key advancements in exposure science, including integrating measured exposure data and probabilistic modeling techniques that improve the accuracy of safety evaluations. Attendees will gain a deeper understanding of why exposure-driven approaches are essential for modern risk assessment.

Presentation: Oral



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Characterizing uncertainty in NAM-based safety assessments

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Significant progress in the development and application of NAM based methodologies within tiered next generation risk assessment (NGRA) frameworks is currently underway, with publications highlighting both the protective nature of NAM based toolsets against current *in vivo* POD, and where available, human benchmark exposures while also developing capability for hazard characterization. The ASPA framework expounds on these previous tiered strategies providing structured guidance to enable implementation in a directed manner, to speed up the adoption and strengthen the transparency and robustness of the assessor's decision making and reporting. The approach provides a progression from simple high throughput *in silico* and *in vitro* approaches towards more mechanistic complex models and systems models. This development is being guided by case studies to evaluate the logic flows and potential gaps. A critical question arising at decision points in the assessment workflow is whether the information is sufficient for the assessment to be conclusive. At the end of Tier 1 this question tries to assess whether safety assessments based on non-animal data can "provide technically reliable information" that is relevant to the understanding of human biology and health protective for the endpoint of concern.

Understanding if an assessment is conclusive requires a clear evaluation of the variability and uncertainty across both the NAM based exposure and hazard pillars, including how to characterize (and combine) the uncertainty from different sources of evidence under a weight of evidence approach. To achieve this an uncertainty framework, based on EFSA's uncertainty guidance, has been proposed that supports the ASPA and provides the transparency in the characterization and quantification of the uncertainty. Based on the problem formulation this approach tries to identify the sources of uncertainty across the different ASPA pillars, such as those from prior evidence or data and those coming from models and those related to variability at both a data generation level and at a population level. The uncertainty framework proposed describes these elements and following an assessment of their impact, weights and assesses these uncertainties and how they impact the conclusion at a combined level. The talk will provide examples of approaches used to explore and address some of the characterized uncertainties in both the NAM *in vitro* data and PBPK models from different case studies undertaken and how they have been integrated as part of an iterative process and used to determine the adequacy of the conclusion including the use of bioactivity exposure ratios.

Presentation: Oral

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NAMs beyond hazard: Exposure-driven toxicology data generation strategy that minimizes animal testing for risk evaluation of pesticides

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With the volume of data required for global pesticide registration, can this wealth of information be utilized to reduce the burden of data review by regulatory authorities, accelerate time to market, and maintain global safety standards? This presentation will explore a pathway that begins with exposure assessment and leverages existing toxicological data. Approaches to estimate dietary and worker exposures will be discussed, focusing on quantitatively evaluating human health risks. Hazard data will be drawn from databases containing extensive toxicological and data and references doses, which can then be refined by pesticide type (e.g., insecticide, fungicide, herbicide) and further categorized by chemical class or mode of action. By evaluating both exposure and hazard, regulators can conservatively assess potential risks associated with specific exposure scenarios. Risk assessment can then be refined, first through exposure analysis and subsequently by targeted animal testing designed to address specific exposure conditions.

Presentation: Oral



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Use of GenAI tools to support regulatory application: ANVISA experience

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Anvisa has been progressively advancing a data- and AI-driven organizational transformation to enhance analytical maturity, data interoperability, regulatory innovation, and transparency. This strategic direction aligns with the 2024-2027 Strategic Plan, particularly Strategic OKR 6, which emphasizes the intensive use of data to optimize regulatory processes and improve institutional efficiency.

The rapid evolution of digital technologies, coupled with the increasing volume and complexity of data, presents new challenges for health regulation. To address these challenges, Anvisa recognizes the need for advanced analytical solutions capable of supporting regulatory decision-making and operational efficiency.

In this context, several initiatives are either underway or being planned to leverage data and artificial intelligence in support of the Agency's mission. One key initiative is the adoption of Generative AI tools to facilitate the daily work of Anvisa's staff across different areas. This effort began in 2023, leading to the delivery of multiple chatbot solutions in 2024, which paved the way for more robust AI-driven solutions, including enhancements to the Generative AI tools themselves.

Following an initial phase of validation and deployment, Anvisa has entered a second phase focused on acquiring the necessary infrastructure to ensure optimal model performance, as well as advancing the adoption of more modern Generative AI models. This phase aims to improve both solution quality and user experience, reinforcing Anvisa's commitment to leveraging AI for regulatory excellence and strengthening its role as an innovative and data-driven regulatory authority.

Presentation: Oral

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Language-driven histopathology predictions for enhanced digital pathology

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Predicting organ-specific toxicity remains a core challenge in regulatory science, typically relying on molecular structures and experimentally derived endpoints. Traditional computational methods, such as quantitative structure-activity relationships (QSAR), employ handcrafted molecular descriptors or graph-based features, often neglecting the context of chemical exposure (Klambauer et al., 2023). Recent advances in generative AI and large language models (LLMs) offer novel opportunities to model toxicity by capturing experimental contexts directly from text (Tong et al., 2024).

As toxicology embraces the 3Rs (Replacement, Reduction, Refinement) through *in silico* methods, we propose language-driven models encoding experimental descriptions into textual features. Instead of conventional molecular encoding (e.g., ECFP fingerprints or SMILES-based embeddings like MolFormer), we explore toxicology study descriptors as inputs to an LLM.

We applied this approach on the large-scale Open TG-GATEs dataset, formulating lesion prediction (presence of liver or kidney lesions) as a classification task, comparing language-driven methods against molecular predictors. We evaluated different text encoding strategies, from general embeddings to pretrained LLMs, using descriptors such as compound identity, dose, and duration. These embeddings were integrated with ensemble machine learning classifiers, trained and assessed following predictive biomarker identification protocols inspired by the MAQC II Consortium.

We tested five modeling strategies: two molecular structure-based models (traditional QSAR and SMILES transformers), two text-based models using engineered features or biomedical embeddings, and a novel context-aware LLM method in zero-shot mode. Models were rigorously evaluated across 1,674 unique treatment conditions via nested cross-validation (Outer-Purged, Inner-Stratified) to assess generalization.

Notably, text-based models and the zero-shot LLM achieved high predictive accuracy without explicit molecular structure input, highlighting that experimental context (e.g., dosing, pharmacological class) carries predictive signals structure-based models miss.

Recent developments in multi-task LLMs tailored for therapeutic and safety prediction (Zambrano et al., 2024) further support our findings, emphasizing advantages when LLMs integrate domain-specific context. This paradigm shift toward context-rich, AI-driven toxicity models provides practical new approaches supporting the 3Rs, paving the way toward reliable, animal-free predictive toxicology strategies.

Presentation: Oral



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Enhancing understanding of engineered nanomaterial reproductive toxicity using new approach technologies

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Unique characteristics of Engineered Nanomaterials (ENMs) and other nanosized particles require specific considerations in order to identify and characterize their impact on human health. New Approach Methodologies (NAMs) offer both advantages as well as challenges to address this information gap and in this presentation the use of *in vitro* systems to provide Next Generation Risk Assessment (NGRA) examples for reproductive and developmental neurotoxicity of ENMs will be discussed. Two distinct classes of ENMs have been tested including silver nanoparticles (AgNPs) as well as quantum dots (Q-dots). One characteristic of these particles is that they frequently have high surface to volume ratios and have antimicrobial properties which allow them to have wide applications in medical, food packaging and clothing applications (AgNPs). Quantum dots have wide applications for use in medical imaging and tracing. These same characteristics and particle shape and size that make them so useful have also made them more challenging to characterize. In this talk we will share several key risk factors that have been evaluated for these particles *in vitro*. These factors include strain and sex-dependent differences and size and particle coatings. In addition, these assessments were done at various developmental timepoints using both proliferating and/or differentiating 3D culture systems. Several culture systems were used including human neural progenitor cells (hNPCs) (hNP1 and NSC-H14, respectively from female and male cell lines) and micromass neuronal cell cultures originating from two different strains of mice (C57BL/6 and A/J). We determined that the percentage of cells differentiating across a 7-day assessment period correlated best with adverse impact from larger AgNPs (110 nm Ag Citrate and Ag PVP coated particles). Significant effects of particle coating, size and window of developmental differentiation were critical factors in prediction of impact. A factor in quantitatively evaluating these conditions and particles was the use of dosimetrically adjusted methods to determine improved exposure assessment context for our analyses of developmental neurotoxicity. The studies on AgNPs were initiated after observations on lung and liver toxicity from *in vivo* studies showed accumulation of these particles in the brain. Results from both nominal and dosimetric dosing showed neuronal cell death increased in a dose-dependent manner at later time points that coincided with differentiation stages of development in both strains. Using the dosimetric dose, neuronal cultures were more sensitive to smaller particles, despite less uptake of Ag in smaller particles (20 nm) than the larger sized 110 nm ENMs. An adverse outcome pathway frame was used to organize these results. The extrapolation of these observations with *in vivo* assessments using NGRA approaches will conclude this talk.

Presentation: Oral

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Case study: Prioritization of chemicals using high-throughput testing

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Chemicals in Europe are regulated based on their annual production volumes and standard test requirements increase with volume. As a consequence, it is estimated that the toxicological properties of only some 600-1000 compounds are very well characterized. Toxicities of the majority (i.e., thousands) of presumably low-tonnage compounds, remain unknown. We will present a case study that is a screening approach to prioritize compounds that cause systemic toxicity after repeated chronic exposure. To this end, predominately high-throughput *in vitro* assays as well as kinetic assessments were used, supported by data from *in silico* models. The aim was to produce an Integrated Approach to Testing and Assessment (IATA) for prioritization that will identify low toxic compounds with high reliability, so that further, for instance more mechanistically-driven testing can focus on the suspected hazardous compounds. We selected 66 data-rich organic compounds, which were divided into two categories based on results from subchronic *in-vivo* rodent studies with oral exposure. About half were classified as low toxic compounds – not showing any specific adverse outcomes until about 1000 mg/kg bw/d – whilst the other half were classified as toxic compounds showing specific adverse effects in systemic target organs, below a LOAEL of 10 mg/kg bw/d. Chemical space analysis was conducted to assure that both categories spanned comparable range of structural characteristics and physico-chemical properties. We subsequently explored the applicability of predominately high-throughput assays (Cell Painting Plus phenotypic assessment in HepG2 cells, CALUX U2OS luciferase reporter panel for nuclear hormone receptor activation, ToxProfiler cellular stress response fluorescent reporter panel and high throughput TempOseq transcriptomics in human primary hepatocytes and human RPTEC-hTERT cells) into a prioritization workflow. We have performed concentration response exposures for all 66 chemicals in all test methods and defined BMCs for the various assays. We will focus the presentation on the transcriptomics data and discuss how our testing toolbox deployed within the ASPA assessment workflow and how this data can aid in prioritizing chemicals for further safety assessment and what considerations can help support potentially intricate overall data integration.

Presentation: Oral



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Qualitative and quantitative interpretation of high throughput transcriptomics for SSbD: The interactive TXG-MAPr toolbox

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For SSbD approaches an important question is whether different biological systems are perturbed by chemical exposure and whether such effects are compound class dependent or not. High throughput transcriptomics (HTTr) is a sensitive, cost and time efficient approach to uncover diverse biological perturbations of different cellular test systems that are representative for various critical target organs of toxicity. Qualitative and quantitative interpretation of HTTr data is essential to compare mode-of-action and potency values for biological perturbations and early guidance and decision making. We have established a TXG-MAPr interpretation toolbox for HTTr interpretation. The TXG-MAPr tool is based on weighted gene co-regulation network analysis (WGCNA) of large HTTr datasets from diverse chemical treatment for individual human *in vitro* test systems. We have established the TXG-MAPr tool for both liver, kidney and neuronal test systems. The tool provides comparison with existing HTTr data as well as compound comparison within a new substance class. The TXG-MAPr toolbox allows HTTr data interpretation by non-bioinformaticians including chemists, biologists and regulators. In the presentation the background of the TXG-MAPr tool will be discussed and the application for SSbD will be exemplified.

This work received funding through the EFSA TXG-MAP and TD-TRAQ projects, the Horizon2020 RISK-HUNT3R project (grant agreement 964537) and the Horizon Europe PARC project (grant agreement 101057014).

Presentation: Oral

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An innovative framework integrating NAMs for next generation risk assessment

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A diversity of *in silico* and *in vitro* NAM approaches for chemical safety assessment emerging. These NAMs aim to provide information on toxicodynamics, toxicokinetics and exposure. Practical workflows that integrate NAM data for a risk assessment are missing. The European Horizon2020 ASPIS cluster RISK-HUNT3R project has established an innovative framework on the integration of NAM data to support next generation risk assessment. The framework has been named ASPA: the ASPIS-initiated alternative Safety Profiling Algorithm. This NGRA framework allows systematic and tiered integration of NAM data with respect to exposure, toxicokinetics and toxicodynamics information. The integration is supported by an online reporting application to establish a NAM-based safety assessment report. The ASPA framework will be exemplified by case studies that involve the various stakeholders from NAM developers, industry and regulators. Learnings with respect to applicability domains, NAM datagaps and regulatory acceptance based on an international NAM-based NGRA workshop will be discussed.

This work received funding through the Horizon2020 ASPIS cluster RISK-HUNT3R project (grant 964537).

Presentation: Oral



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Implementation of OECD TG 249 as an alternative to acute fish toxicity testing at the university of Costa Rica

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The Biological Testing Laboratory at the University of Costa Rica established an experimental aquarium approximately five years ago to support aquatic toxicology research. Since its inception, the laboratory has applied the OECD Test Guideline 203 (Acute Fish Toxicity Test) to evaluate the effects of various substances – primarily pharmaceuticals – on aquatic environments. These evaluations have been informed by literature based on river water sampling across Costa Rica. However, this assay requires a minimum of 24 fish per test, leading to a considerable use of live animals.

In an effort to reduce and replace the use of vertebrate animals in toxicity testing, the OECD published Test Guideline 249 in 2021. This alternative method employs a cytotoxicity assay using the RTgill-W1 cell line, derived from rainbow trout gill epithelial cells. The implementation of this assay at our laboratory aims to support the 3Rs principle (Replacement, Reduction, and Refinement) in scientific research. Successful standardization of this method could result in a reduction of at least 100 fish per year by substituting the acute fish toxicity assay with a validated *in vitro* alternative.

This initiative is part of the newly established Alternative Methods Section, the first of its kind in Costa Rica, which is dedicated to developing and promoting non-animal testing approaches for toxicological and biomedical research.

Presentation: Oral

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Leveraging strategic funding and public-private partnerships to establish confidence in NAMs for biomedical research and regulatory testing

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Building confidence in new approach methodologies (NAMs) for biomedical research and regulatory testing requires more than scientific innovation – it demands sustained investment, cross-sector collaboration, and coordinated policy alignment. This presentation will highlight two transformative NIH programs that exemplify how strategic funding and public-private partnerships are being leveraged to accelerate the development, validation, and adoption of human-relevant, non-animal methods.

The Complement Animal Research In Experimentation (Complement-ARIE) program, launched under the NIH Common Fund, seeks to identify and support innovative, human-based technologies that can complement or replace traditional animal models in areas where they are known to be poorly predictive or where human relevance is critical. Complement-ARIE emphasizes multi-disciplinary collaboration and clear translational endpoints to ensure funded projects address defined scientific and regulatory needs.

In parallel, the newly established Office of Research Innovation, Validation, and Application (ORIVA) within the NIH Office of the Director is designed to provide centralized leadership in identifying, validating, and promoting human-based technologies across NIH and with key federal and international partners. ORIVA serves as a nexus for policy alignment, stakeholder engagement, and the strategic coordination of funding mechanisms to ensure that emerging NAMs are developed with an eye toward broad acceptance and impact.

Together, these initiatives reflect a paradigm shift in how the U.S. government approaches scientific innovation – by integrating hypothesis-driven next generation safety science with policy needs and international harmonization efforts. The presentation will outline best practices, identify common barriers, and offer insights into how targeted investments and cooperative frameworks can streamline validation and qualification processes. Ultimately, these programs serve as models for how to create robust, scalable pathways for the global adoption of NAMs in both research and regulatory contexts.

Presentation: Oral



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Translating experience into progress: Advancing regulatory acceptance of NGRA

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The transition to Next Generation Risk Assessment (NGRA) offers a path toward regulatory systems that are more protective, human-relevant, and less reliant on animal testing. However, broader regulatory acceptance of NGRA requires more than just new scientific tools – it demands a deliberate shift in how risk is characterized, and decisions are made.

U.S. federal agencies are advancing this transformation through coordinated efforts. At NIH, the Complement Animal Research In Experimentation (Complement-ARIE) program and the newly established Office of Research Innovation, Validation, and Application (ORIVA) are prioritizing the development and implementation of human-based research technologies. Concurrently, NIEHS and NICEATM are leading national efforts to develop, validate, and promote new approach methodologies (NAMs) in close collaboration with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).

The FDA's 2024 Advancing New Alternative Methods roadmap further reinforces this direction by outlining a clear regulatory strategy to adopt NAMs where scientifically appropriate. Together, these initiatives are enabling a shift from traditional animal-based predictions toward fit-for-purpose, exposure-led, and mechanistically grounded safety assessments. This presentation will explore how these aligned efforts are creating the scientific and policy infrastructure needed to support regulatory acceptance of NGRA.

Presentation: Oral

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A perspective from Brazil

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In Brazil, the law that regulates the use of animals in teaching, testing and research is Law nº 11.794 from October 8th, 2008. It covers filo *Chordata*, subfilo *Vertebrata*, except man. Even though it is a new law, we plan to review and update it.

The Council for the Control of Animal Experiments in Brazil (CON-CEA) is starting to collect official data on the number of animals used in research and testing. The 3Rs are in our law, so we must follow them, and no research project with animals is approved if researchers do not prove that there are no alternatives for that research. Disobedience to the law implies penalties. New projects must be evaluated by an Ethical Committee, and to be approved it has to have a strong justification, to observe humane endpoints, avoid animal suffering and many other items to preserve animal welfare, when no alternative is available.

But, independently of the law, there are many workshops, seminars, and other activities to try to sensitize people on the 3Rs. I dare say that students are very much interested in using alternatives instead of animals. Nowadays we have many initiatives to help and motivate people to work with alternatives from the government, universities, and financial agencies. Of course, we always need more help.

Presentation: Oral



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Regulation of animal experiments in India

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The Committee for Control and Supervision of Experiments on Animals (CCSEA) was constituted by the Government of India in 1968 under chapter IV of the “Prevention of Cruelty to Animals” (PCA) Act, 1960. In 1998, the rules were re-notified in the Gazette of India as the “Breeding of and Experiment on Animals (Control and Supervision) Rules”. CCSEA was given a mandate to execute these “Rules” scrupulously.

Every establishment carrying breeding and/or experiments on animals must apply for permission to do so after seeking such registration from the CCSEA. The first step towards registering the Animal Facility with CCSEA is to constitute an Institutional Animal Ethics Committee (IAEC). The IACUC/IAEC should have five members as follows – a biological scientist, two scientists from different biological discipline, a veterinarian involved in care of animals, scientist In-Charge of the Animal Facility. The Head of the organization, or his Nominee, could be the Chairperson. CCSEA nominates a Main Nominee, scientist from outside the institute, socially aware member and link nominee.

It is the responsibility of the Chairperson to conduct the proceedings of the meetings of the IAEC strictly as per the guidelines of the CCSEA. The IACUC/IAEC is required to meet twice a year irrespective of whether there are animal study proposals for discussion or not. IAEC is also responsible for monitoring the functioning of the Animal Facility, to ensure that the experimentation complies with the rules and regulations of the CCSEA and also ensure ethical animal care and welfare.

The member Secretary has to ensure that all animal study proposals are written in lay language, enabling all members of the IACUC/IAEC to properly understand them. All experiments involving the use of laboratory animals up to the phylogenic level of rodents (mice, rat, hamster and rabbits) can be reviewed and approved by the IACUC/IAEC. Animal use above rabbits is to be recommended by the IACUC/IAEC and to be forwarded to the CPCSEA, New Delhi for approval of the animal use.

Although international regulations for animal experimentation may differ, the goal is always to minimize the use of animals in research and to provide best possible humane care and welfare to the animals through the laws which regulate the animal research in the respective country.

Presentation: Oral

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From printing to perfusion: Tumor-on-chip systems for 3R-aligned cancer research

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Understanding cancer invasion and metastasis requires advanced *in vitro* models that replicate key features of tumor microenvironments. In this study, we present a chaotic bioprinting approach to fabricate prevascularized tumor niches, enabling the integration of cancer spheroids with vascular-like microchannels in a single construct. Using MDA-MB-231 breast cancer cells, we demonstrate that multichannel scaffolds created via chaotic bioprinting significantly enhance cancer cell migration and alignment compared to traditional solid constructs. Migratory fronts in multichannel filaments extended up to four times farther ($1593 \pm 884.4 \mu\text{m}$) and exhibited higher directional alignment (88%) by day 20.

In a second stage of the project, we used 3D printed Mili-fluidic systems (fabricated in stereolithographic 3D printers) to incubate the bioprinted tumor niches under continuous perfusion. We were able to sustain high cell viabilities in this integrated tumor-on-chips for 4 weeks.

Gene expression analysis revealed accelerated epithelial-to-mesenchymal transition in multichannel scaffolds, with earlier peaks in N-cadherin expression and elevated levels of vimentin. Enhanced migration was accompanied by increased cellular proliferation, as evidenced by higher Ki-67 expression. Importantly, the absence of hypoxia in migratory fronts underscores the role of microchannel architecture in promoting sustained, non-hypoxic migration.

This work establishes chaotic bioprinting as a versatile platform for creating complex tumor models with enhanced functionality, offering new opportunities for studying cancer dynamics and optimizing anti-metastatic therapies. Moreover, the integration of chaotic printed tumor-niches and mili-fluidic platforms yielded promising platforms to conduct cancer research in full alignment with the 3R concept.

Presentation: Oral



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Threshold of toxicological concern (TTC) – From human health to environment – refining ecological TTC using enhanced database of fragrance chemicals

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The threshold for toxicology concern (TTC) is a well-established and accepted concept in human health safety. Its purpose is to rapidly screen and assess data-poor materials and mixtures by establishing an exposure concentration below which negligible risk is expected. The ecological threshold of toxicologic concern (ecoTTC) was created back in 2015 (Belanger et al. 2015) and proposed as a means to summarize larger amounts of ecotoxicity data with the purpose of Predictive No Effect Concentration (PNEC) extrapolation. Leveraging database with computational tools (Ecotoxicological Information System, EnviroTox) ecoTTC allows the prediction of 5th percentile PNEC for ecosystems using ecotoxicity data extrapolated to individual PNEC for individual compounds. Since TTC has been used by Research Institute for Fragrance Materials (RIFM) to screen fragrance materials in human health assessments, an effort began to develop an ecoTTC for fragrance materials. A review of available ecotoxicological data in the RIFM database as well as additional literature including Europea Chemicals Agency (ECHA) registration database was used to derive ecological Thresholds of Toxicologic Concern (ecoTTC) and Chemical Toxicity Distributions (CTD). For this project the following ecoTTC assessments and comparisons were made: Material alone versus supplementation by EnviroTox; groupings based on chemical class, Verhaar Mode of Action (MOA) and MOATOX MOA classification, groupings based on various bands of log Kow, etc. In addition, US-like and European-like ecoTTC assessments were evaluated. A total of 1094 ecotoxicity studies of all types were summarized covering 424 fragrance materials and results from analysis are going to be reported during the presentation.

Presentation: Oral

Poster Abstracts

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Advantages of collaborative approach in psychiatry

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Aim: Psychiatry and Paediatrics joint clinic aims to highlight the advantages of this collaborative approach to address the complex, multifaceted needs of patients, including better patient satisfaction and holistic treatment plans.

Background: Mental health and physical health are deeply interconnected, yet healthcare systems often treat them in isolation leading to fragmented and poorly coordinated service and poor-quality patient experiences. We identified a need for joint Paediatric and Child and Adolescent Mental Health Services (CAMHS), integrating into a collaborative model of care.

In the UK, nearly three quarters of young people with mental health difficulties also have physical symptoms, whilst children with any physical complaint have an 82% higher probability of developing a significant mental health condition.

To address this issue, North West London collaboration between the paediatric team and Child and Adolescent Mental Health Services (CAMHS) team proposed the creation of the “Mind and Body Clinic”: an integrated physical and mental health clinic. We aim to highlight the advantages of this collaborative approach, including holistic treatment plans, education and early direction towards appropriate services.

Methods: The “Mind and Body Clinic” was created in May 2024 and operates as a monthly afternoon clinic. Paediatricians, who had assessed children and young people up to 18-years-old and have appropriately excluded organic causes for their symptoms could be referred. A consultant paediatrician and a consultant Child & Adolescent Psychiatrist review the referrals, and accepted patients are booked into the clinic. A paediatrician and CAMHS psychiatrist at either registrar or consultant level jointly review patients in 1-hour-long appointment slots. A prospective survey was conducted to evaluate the clinic. Patients, their parents, and medical staff completed a feedback survey via an online form after each clinic appointment. After a pilot trial of 4 months, data from the survey were analyzed by the paediatric and CAMHS teams.

Results: As of December 2024, 25 patients have been referred to the “Mind and Body Clinic”. 20 patients were accepted, out of which 15 have been reviewed in clinic so far. 10 patients and 15 parents completed the feedback survey. Their responses to a series of evaluation questions for the clinic ranked on a 5-level Likert scale. All participants were either extremely likely or likely to recommend the clinic to their friends and family. Free-text responses were appreciative of the service.

Conclusions: Patients and their parents who have been reviewed in the “Mind and Body Clinic” think it has been effective in addressing their health needs. They also found it helpful seeing both a paediatrician and a CAMHS psychiatrist in the same clinic.

This has been a successful pilot of an integrated physical and mental health clinic and provides a platform for its continuation and further evaluation.

Presentation: Poster



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Applicability of high throughput new approach methods to classify compounds as being specifically toxic to target organs after repeated exposure

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In order to achieve the replacement of *in vivo* animal studies, it is necessary to learn how to use New Approach Methods for decision making in regulatory risk assessment, for example for the classification and labeling of compounds.

This presentation will provide a synopsis of the recent findings from a Riskhunt3R case study, which investigates the applicability of high-throughput methods, including transcriptomics, cell painting and reporter cell assays, for distinguishing compounds currently classified as STOT-RE (specific target organ toxicity after repeated exposure). To this end, a training set of 61 compounds has been assessed. These compounds have been shown to exhibit either LOAEL values below 10 mg/kg bw/d or no adverse effects up to the maximal tested dose of 1000 mg/kg bw/d in their existing *in vivo* studies with repeated exposure.

The individual NAM methods already distinguished both categories fairly well and machine learning methods are under investigation to assess the added value of their combination. The concordance of the individual methods, the most predictive NAM features as well as their underlying biological processes will be presented.

This information will be implemented into the ASPA (ASPIS-initiated alternative Safety Profiling Algorithm) framework, to further improve this Next Generation Risk Assessment tool.

Presentation: Poster

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Expert consensus on the therapeutic use of cannabis in veterinary medicine: A Delphi study

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The integration of cannabis-based therapeutics into veterinary medicine represents a paradigm shift with the potential to revolutionize the management of complex conditions in companion animals. This study employs the Delphi method to establish expert consensus guidelines, addressing the critical need for standardized practices in the administration of cannabis to dogs and cats.

Background: Despite the increasing interest in veterinary applications of cannabis, the field is hindered by a lack of uniform guidelines and regulatory clarity. This research seeks to contribute to these voids by providing expertise-based recommendations that align with the principles of One Health, emphasizing the interconnectedness of human, animal, and environmental health.

Methods: A panel of esteemed veterinarians and researchers, recognized for their expertise in cannabis therapeutics, participated in a series of Delphi rounds. This iterative process involved structured surveys designed to distill collective expert opinion into actionable guidelines, ensuring a comprehensive and multidisciplinary approach.

Results: The consensus delineated several key areas: (1) Therapeutic indications, particularly for chronic pain, epilepsy, and anxiety, where cannabis demonstrates significant potential; (2) Dosage and administration protocols, advocating for individualized therapeutic strategies; (3) Safety considerations and adverse effect management, highlighting the necessity of rigorous monitoring; and (4) A comparative evaluation of CBD and THC efficacy in diverse clinical scenarios.

Discussion: The findings underscore the transformative potential of cannabis within veterinary practice, advocating for its informed and responsible use. The consensus adds to a foundational framework that empowers veterinarians to integrate cannabis therapeutics effectively, while also identifying areas requiring further empirical investigation to refine and expand current guidelines.

Conclusion: This Delphi consensus constitutes a pivotal advancement in the domain of veterinary medicine and One Health, offering a robust foundation for the integration of cannabis-based therapies. By synthesizing expert insights into coherent guidelines, this study not only facilitates clinical decision-making but also contributes to future regulatory and research endeavors. As the landscape of veterinary therapeutics evolves, continued validation through rigorous clinical trials will be imperative to fully realize the potential of cannabis in enhancing animal health and fostering holistic well-being.

Presentation: Poster



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Real-world evidence on cannabis therapeutics: An integrated digital platform approach with One Health perspective

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Introduction: The recent implementation of State Law 17.618/23 in São Paulo, authorizing cannabis distribution in the public health system, demands robust evidence on efficacy and safety. This study aims to address this need through an innovative digital platform approach, integrating human and veterinary medicine within a One Health framework.

Objectives: Develop a digital platform to collect and analyze Real-World Data (RWD) on cannabis use in various medical conditions; generate scientific evidence to inform public policies and clinical practices; contribute to the safe, evidence-based expansion of medicinal cannabis use; promote education among health professionals and the public; and implement a traceability system for the supply chain.

Methodology: Using the www.caonabico.com platform, the study collects RWD via online questionnaires over 24 months, in three phases: Veterinary Cannabis Therapeutics, Human Cannabis Therapeutics, and Social Impact Assessment. The sample includes 200 participants, with data collected every 60 days for 6 months per participant. The platform incorporates AI algorithms for analysis and blockchain for traceability, ensuring anonymity and data security.

Preliminary results: Preliminary analysis of 46 questionnaire responses revealed demographic and cannabis use data. The average age of human participants is 45 years, with 58% female. The most commonly treated conditions include chronic pain (35%), anxiety (30%), and epilepsy (15%). In animals, the main conditions are chronic pain (40%) and anxiety (25%). 70% of human users and 60% of animal caregivers reported significant symptom improvement. Mild side effects were reported in 20% of cases, mainly drowsiness and dry mouth.

Conclusion: Preliminary results suggest significant therapeutic potential of cannabis in various conditions, both in humans and animals, with a favorable safety profile. This project aims to create a robust infrastructure for collecting and analyzing data on medicinal cannabis, adopting an innovative One Health approach. By integrating advanced technologies with scientific inquiry and social impact initiatives, the study seeks to transform clinical practice, inform public policies, and contribute significantly to scientific knowledge in this rapidly evolving field.

Expected impact: This study is expected to provide crucial evidence to guide the safe and effective use of cannabis products, contributing to the formulation of evidence-based public policies and advancing medicinal cannabis research in Brazil. Additionally, the project aims to promote education and awareness about the therapeutic use of cannabis, potentially benefiting patients, healthcare professionals, and the related economic sector.

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A modern approach to assessing the human safety risk of agrochemicals without unnecessary animal testing

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Advancements in the technologies, mechanistic research and approaches to data analysis have expanded the portfolio of new approach methods (NAMs) for the safety assessment of agrochemicals. Building on this progress an approach was developed to demonstrate a modern scientifically sound and robust strategy that applies appropriate and flexible exposure and effects characterization without chemical-specific animal testing. In place of data generated from animal testing, alternative lines of evidence were compiled to reliably assess and address the risk, uncertainties, and deficiencies in data and its interpretation with equivalent confidence as do the currently accepted test guidelines and meet the regulatory needs of the Agencies. This presentation will provide an example of this approach with a new ACCase inhibitor. A selection of chemical analogues for read-across assessment, identified based on pesticidal mode of action, chemical class, structural similarities, and toxicological properties, were compared to the available bridging data for the new ACCase inhibitor to ensure consistency of response to support the read across. Transcriptomics analyses were conducted to further assess the bioactivity similarities between chemicals. The lines of evidence used included an evaluation of use pattern(s), exposure scenario(s), pesticidal mode-of-action, physicochemical properties, metabolism, toxicokinetics, toxicological data including mechanistic data, and an evaluation of the reliability and consistency of the toxicological response(s) between the chemical of interest and read-across analogs. The identified endpoints for safety and risk assessment were supported by the additional lines of evidence. Following a read across assessment of the biologically similar ACCase inhibitors, this human safety and risk assessment indicates that the planned use profile for the new ACCase inhibitor does not present a significant human health and safety risk. This presentation will highlight the important aspects considered in this weight of evidence assessment to exemplify a process to help determine when sufficient information is available to identify a health protective endpoint for risk assessment for a non-genotoxic agrochemical without performing the OECD Test Guideline toxicity studies. This risk assessment is offered as a modern approach to testing and evaluation, with the intention of addressing risk without unnecessary animal testing.

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Advancing respiratory tract cell culture NAM models for risk-based decision making

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The high cost and large numbers of animals associated with classical *in vivo* toxicology for portal of entry effects from inhalation exposures combined with significant anatomical and physiological differences between animal and human respiratory systems make the translation of *in vivo* inhalation results into predicted human effects difficult. Human lung organotypic cultures are currently being used to assess direct toxicity of inhaled chemicals currently in a case-by-case basis, however, an OECD guideline will facilitate wider uptake by providing clarity on what is required for regulatory acceptance.

The objective of this project is to develop an *in vitro* aerosol chemical screening assay protocol for risk-based decision-making. To achieve this aim and work within a tiered risk assessment, a panel of 10 chemicals relevant to human inhaled risk from aerosols were selected, covering a range of toxicities, modes of action, and having known high quality rodent inhalation toxicity data available for comparison of point-of-contact toxicity in the lung.

This presentation will provide an example of a tiered approach for inhalation risk assessment of aerosol delivered chemicals. The following steps were/will be taken to achieve this aim: 1) Perform dose-range finding studies to determine the *in vitro* toxicity of aerosol chemicals and the maximum tolerated dose for each chemical following acute exposure using 3D rat and human airway tissues and monolayer primary human airway epithelial cells in two laboratories. 2) Perform repeat chemical application experiments over a 14-day period using rat and human airway tissues. 3) Evaluate effect of acute and repeat application of aerosol to 3D human airway tissues. 4) Undertake respiratory tract dosimetry model (MPD and CFD) to compare existing rodent aerosol delivered datasets. 5) Determine reproducibility of chemical toxicity endpoints using human airway tissues reconstructed from 3 donors. 6) Develop prediction model to test unknown chemicals.

We present data from the evaluation of acute (4 h exposure following direct application to a range of concentrations) and chronic (14 day repeat application) toxicity and point of departure of the panel of chemicals, across two laboratories (Charles River, UK and MatTek, USA) using harmonized. The data gathered so far show that this tiered approach has the potential to allow prediction of human hazard classifications for inhalation risk. This work is part of a larger program which also includes repeat dose responses, aerosol vs liquid applications, and donor differences, with the eventual goal of generating a prediction model for inhalation risk of aerosol chemicals. Evaluation of the *in vitro* toxicological platforms for aerosol chemical screening aligns with the strategic goal by regulatory bodies to develop reliable, translatable, predictive, and cost-effective alternative methodologies to animal testing.

This work is funded by the American Chemistry Council's Long Range Research Initiative.

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Regulatory qualification of microphysiological systems (MPS) as drug development tools: The NIH-FDA Translational Centers for MPS

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Current methods used to predict safety and efficacy of candidate drugs accounts for as much as 90% failure rate. Approximately 30% of drugs have failed in human clinical trials due to adverse reactions, and another 60% fail due to lack of efficacy. A number of these failures can be attributed to poor predictability of human response from animal and 2-D *in vitro* models currently being used in drug development. Recent systematic studies on the predictive value of animal models have demonstrated a poor correlation between animal data and human outcomes owing to substantial interspecies differences in key disease pathways and disease-induced changes in gene expression profiles, highlighting the critical need for alternative methods to model complex human-relevant conditions. To address this challenge, the NIH Tissue Chips or Microphysiological Systems (MPS) program have been supporting the development of MPS as better risk assessment tools that can provide more reliable readouts of toxicity and efficacy of candidate therapies. MPS are bioengineered 3-D microfluidic platforms utilizing chip technology and human-derived cells and tissues that are intended to mimic tissue cytoarchitecture and functional units of human organs and systems. In addition to drug development, these microfabricated devices are useful for modeling human diseases, and for studies in precision medicine and environmental exposures. By emulating human physiology, these chips have the potential to increase the predictive power of preclinical modeling, which in turn will move the pharmaceutical industry closer to its aspiration of clinically relevant and ultimately animal-free drug discovery. MPS as novel preclinical modeling platforms offer improved clinical predictions of human response, provide a more efficient approach to mechanistic investigation, early safety liability screening and even more translationally relevant modeling of drug distribution and metabolism. Near term regulatory approval of these human-relevant platforms will fill gaps in current capabilities for assessing important properties of disposition, efficacy, and safety liabilities. The recent passage of the FDA Modernization Act 2.0 will only hasten this process. Towards this end, the National Institutes of Health (NIH) led by the National Center for Advancing Translational Sciences (NCATS) have established Translational Centers for MPS (TraCe MPS) to qualify tissue chips as drug development tools. These Centers aims to support research that will accelerate the translational use of MPS in drug (both human pharmacological and biological products) development through regulatory acceptance and adoption for industrial use, by establishing MPS that are fit-for-purpose for industry needs, have specific defined context-of-use (CoU) in drug development and will be developed with consideration of applicable expectations to achieve regulatory approval. The TraCe MPS program is in partnership with the US Food and Drug Administration (FDA) and the Critical Path Institute. The four TraCe MPS centers are focused on qualification of various MPS platforms developed for liver, kidney, barrier-function, and pregnancy and women's health. This presentation will provide an overview of the NCATS Tissue Chips program, including activities of the TraCe MPS and the process involved in getting MPS qualified as drug development tools.

Presentation: Poster



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Development of an advanced human-based *in vitro* replacement microphysiological system for modelling endometriosis

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Endometriosis is chronic and debilitating condition that affects approximately 10% of reproductive aged women worldwide. It occurs when the endometrium, which lines the inside of the uterus, grows in areas outside of the uterus, such as the peritoneum, ovaries and fallopian tubes. The misplaced tissue can cause severe pain, infertility and other morbidities.

The development of endometriosis is inevitably linked to menstruation, but its pathogenic mechanisms remain poorly understood, despite its prevalence. Historically, the most biologically relevant models of endometriosis are in menstruating non-human primates and in rodents. These carry significant costs and ethical concerns as well as limitations of translating findings to the clinic due to species differences and experimental methodologies such as administration of exogenous hormones and menstrual tissue transplantation. Developing a more accurate and effective model to study endometriosis is essential to advance our understanding of the condition and establish better therapies.

We aim to develop a sophisticated human-based *in vitro* microphysiological system (MPS) that accurately mimics the cyclical changes of the endometrium under the influence of hormones and fits into the Replacement principle of the 3Rs, thereby answering the ethical, economical and scientific concerns associated with animal experimentation. Our MPS consists of three interconnected modules, each dedicated to simulating different aspects of the female reproductive system, with emphasis on endometriosis. The first module concerns a programmable pump system that delivers reproductive hormones at different concentrations according to each stage of the menstrual cycle to a second module, where a human-based 3D model of the endometrium is cultured. Models of typically affected tissue are cultured in the third module, where invasion by the endometrial tissue occurs. Altogether, the MPS is designed for microscopy imaging in a non-invasive manner, but it also allows tissue recovery for study of protein and gene expression.

We find that 3D co-culture models of human endometrium epithelial and stromal cell lines recapitulate features of human menstruation when exposed to different concentrations of estrogen and progesterone. Preliminary data suggests that fibroblast and intestinal cell constructs can be invaded by endometrial cells causing an increment of cell death. This showcases the feasibility of building a biologically relevant non-animal method for the study of endometriosis that can foster research of new drug targets and biomarkers in pharmaceutical, biotech and academic institutions.

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Bioactivity:exposure ratios derived from a systemic NAM-toolbox distinguish between low- and high-risk chemical exposure scenarios

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Integrating New Approach Methodologies (NAMs) for Next Generation Risk Assessment (NGRA) poses significant challenges for complex toxicological endpoints like repeated dose systemic toxicity, due to the variety of mechanisms that can lead to observed toxicological effects. Consequently, a direct replacement of a no observed (adverse) effect levels (NO(A)EL) derived from sub-chronic and chronic repeated dose animal tests is not feasible. Instead, a promising strategy involves deriving surrogate points of departure (PoD) for systemic toxicity from an *in vitro* battery designed to provide bioactivity measures that are not linked to specific hazards but are protective of human health. The context of use for this toolbox is defined as a low-tier approach for conducting systemic toxicity risk assessments that would achieve protection of human health.

The toolbox comprises physiologically-based kinetic (PBK) models to predict internal exposures and bioactivity NAMs designed to cover a broad range of toxicity modes of action. The *in vitro* battery includes high-throughput transcriptomics (HTTr), high-throughput phenotypic profiling (HTPP; cell painting), *in vitro* pharmacology profiling (IPP), and cellular stress panels (CSP), from which PoD are obtained. This toolbox and workflow were evaluated using 38 chemicals and 70 consumer exposure scenarios. The output of the toolbox is the calculation of a bioactivity:exposure ratio (analogous to a margin of internal exposure) for each chemical-exposure scenario. A comparison between the NAM-based PoDs and traditional *in vivo* PoDs identified from sub-chronic and chronic animal studies was performed to further investigate the performance of the NAM-based systemic safety toolbox.

Bioactivity:exposure ratios (BERs) were calculated using the lowest PoD across all bioactivity platforms for each chemical and dividing them by the plasma C_{max} estimates for each chemical exposure scenario. In previous work, a BER of 110, 11, or 2.5 or greater was needed to confidently conclude a low-risk outcome, depending on the confidence in the PBK model (parameterized with *in silico* only, *in vitro*, or human *in vivo* data, respectively). This decision model correctly identified 43 out of 46 high-risk exposure scenarios as uncertain risk (BER < 11) and 6 out of 22 low-risk exposure scenarios as low risk. This means that the toolbox is protective for 93% of the exposure scenarios but only correctly identifies low-risk scenarios for 27% of the cases when only using NAM data (for the remaining 73%, the toolbox would classify the scenarios as uncertain risk). Similar to other studies comparing NAM PoDs with traditional PoDs, we found that for nearly all chemicals, the minimum NAM PoD was more conservative (i.e., lower) than the minimum traditional PoD. Exceptions to this were aspartame and fenbuconazole, where the difference between the traditional PoD and the *in vitro* PoD was under 5-fold.



This work contributes to the growing body of evidence showing that NAM-based safety decisions can be protective of human health and that NAMs can be integrated in a practical and systematic way as part of a tiered approach to enable confident safety decision-making. The approach to evaluating the toolbox also demonstrates that it is possible to assess the performance of a multi-NAM approach by anchoring the evaluation in risk benchmarking.

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***In vitro* evaluation of a veterinary chondroprotective product as an alternative to reduce animal use in osteoarthritis research**

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Osteoarthritis (OA) is a common, chronic, and degenerative joint disease that affects dogs, leading to pain, inflammation, and reduced mobility. This condition significantly impacts the quality of life of affected dogs, causing discomfort and limiting their daily activities [1]. Currently, the management of OA in dogs focuses on alleviating symptoms, as there is no known cure for the disease. Among the most widely used chondroprotective agents for managing canine OA are glucosamine and chondroitin sulfate. These compounds are believed to support joint health by promoting cartilage repair and reducing inflammation [2].

However, according to the Brazilian regulatory agency, the efficacy of a veterinary product must be evaluated in species-specific animals that are naturally affected by or experimentally induced to develop the disease. This requirement leads to the use of a substantial number of

dogs for the evaluation of a single product. To address this ethical concern and reduce the number of dogs used in such studies, we conducted *in vitro* tests to assess the response of cultured canine cells to a veterinary chondroprotective product (Condroton®) containing glucosamine, chondroitin sulfate, and methylsulfonylmethane.

In this study, mesenchymal stem cells obtained from a commercial cell bank were cultured *in vitro* and exposed to the chondroprotective product at a concentration of 12 mg/mL, as recommended by the manufacturer for animal consumption. We evaluated cell viability using the MTT assay and measured cytokine levels (interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1)) in the cell culture supernatant using a Luminex assay (Milliplex CCYTOMAG-90K). The results demonstrated that the product was non-cytotoxic (cell viability = $104 \pm 5\%$). Moreover, cytokine analysis revealed a significant reduction in IL-8 levels (control: $46,969 \pm 285$ pg/mL; treatment: $8,345 \pm 2,009$ pg/mL; $p < 0.0001$) and MCP-1 levels (control: $32,652 \pm 1,946$ pg/mL; treatment: $22,090 \pm 1,287$ pg/mL; $p < 0.001$).

In dogs with osteoarthritis, cytokines such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) play critical roles in the inflammatory processes associated with the disease. These cytokines are typically found in elevated concentrations in the synovial fluid of affected joints [3]. The reduction in these cytokines observed in our study suggests that the veterinary product may help alleviate inflammation, providing a preliminary proof of concept for its anti-inflammatory properties.

This result marks a significant step toward demonstrating the potential of *in vitro* tests to reduce the reliance on animal testing in the evaluation of veterinary products. By establishing such alternative approaches, we can advance research in veterinary medicine while adhering to ethical and regulatory considerations.

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iPSC-derived brain organoids to study the mechanisms underlying disease variability in patients with SYNGAP1-related disorder (SYNGAP1-RD)

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Neurodevelopmental disorders (NDDs) affect approximately 1 in 6 children in the United States, with SYNGAP1-Related Disorder (SYNGAP1-RD) being a rare genetic cause of NDD. SYNGAP1 encodes Synaptic Ras GTPase-activating protein 1, a key regulator of biochemical signaling in neurons. Mutations in this gene disrupt dendritic and synaptic maturation, leading to a spectrum of clinical manifestations, including autism spectrum disorder (ASD), intellectual disability (ID), and epilepsy. The severity and presentation of these symptoms vary widely among individuals, and the cellular and molecular mechanisms driving this phenotypic variability remain poorly understood. Additionally, existing animal models of SYNGAP1-RD do not accurately recapitulate the diversity of clinical presentation seen in patients. Furthermore, no disease-specific treatments currently exist for SYNGAP1-RD, underscoring the need for advanced research models.

To address these challenges, we propose using induced pluripotent stem cell (iPSC)- derived brain organoid models as a high-throughput, human-relevant alternative to traditional animal testing. iPSC-derived brain organoids allow researchers to study neural morphology, analyze

gene expression patterns associated with intellectual disability and epilepsy, and assess electrophysiological properties in a patient-specific context. This approach not only offers scalability and precision but also bypasses the ethical and translational limitations of animal models.

Our lab has previously established and characterized an iPSC-derived brain organoid model using the autism risk gene CHD8 and copy number variation, 16p11.2 deletion, demonstrating its utility in modeling neurodevelopmental disorders [1]. In this study, we reprogrammed human peripheral blood mononuclear cells (PBMC) from a group of patients with SYNGAP1-RD and family members who serve as controls. Three SYNGAP1 and three control lines have been generated and underwent a thorough characterization: karyotyping, pluripotency and mycoplasma testing. One of the SYNGAP1 lines showed a duplication in chromosome 1 due to the reprogramming (a common side effect of iPSC generation) and therefore will be repeated. The neural progenitor differentiation of two SYNGAP1-RD lines and two corresponding controls, which have successfully achieved the quality control validation steps, is currently ongoing and has been characterized via immunostaining with neural markers and high-content confocal imaging. Our ongoing work is focused on SYNGAP1-RD -specific differences between the organoids with the focus on synaptogenesis and electrophysiological activity.

Our goal is to use this platform to define the cellular and molecular mechanisms underlying phenotypic variability and pave the way for effective therapeutic interventions. iPSC-derived brain organoids offer a scalable and reproducible model system, providing a robust platform for advancing research on SYNGAP1-RD and other rare neurodevelopmental disorders. This model holds promise for improving our understanding of disease mechanisms and expediting the development of targeted treatments, ultimately enhancing patient outcomes in the field of neurodevelopmental research.

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The Netherlands transition to animal-free innovations by generating localized hubs: An example given by Amsterdam

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The Netherlands has both a top-down and a bottom-up attitude to accelerate the transition to animal free innovations. Localized hubs have emerged across the Netherlands that coordinate efforts and share experiences in integrating animal-free innovations into research environments, thereby supporting a broad adoption of these advancements. In this way, the Netherlands work together to implement and transition to animal free science. Each hub is a combined area location consisting of universities, medical centers, high schools, and industry in, e.g., Amsterdam, Utrecht, Leiden, Wageningen regions. Here we will showcase the Amsterdam TPI hub. Biomedical and bioinformatics scientists from knowledge centers in Amsterdam, including Amsterdam UMC, Vrije Universiteit Amsterdam, University of Amsterdam and Academic Center for Dentistry (ACTA) meet to discuss what is happening already in Amsterdam regarding animal free innovations and to make plans to accelerate this transition. Action points are divided into theme groups such as education at the undergraduate and post graduate level, supply of human tissue and cells for research, transparency in animal research in Amsterdam, funding acquisition and societal Impact, symposia and networking, organoid models, links with national initiatives, e.g., human disease model technologies consortium (hDMT). Challenges and progress are actively discussed and information shared. This fosters the development of the local network, and in turn encourages interaction on a national scale. By working together locally and nationally we help each other to accelerate the transition to animal free innovations in The Netherlands.

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A sacrificial 3D printed vessel-on-chip demonstrates a versatile approach to model connective tissue pathology

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In microphysiological systems, vasculature is crucial to overcome nutrient diffusion limits and to generate immunocompetent models by allowing transendothelial migration of immune cells in and out of tissue and organ models. However, vasculature is often disregarded due to its complexity to generate and the necessity to integrate flow to emulate the hemodynamic environment. In this study, the aim was to overcome these limitations by combining three-dimensional (3D) printing and Organ-on-Chip (OoC) technology to generate a vessel within a fibroblast-populated connective tissue matrix on-chip. A 3D printed, water-dissolvable structure was incorporated into a multi-organ-chip (MOC) platform during its fabrication and used to generate hollow channels within a collagen/fibrin hydrogel. The flow rates and shear stress within the model were characterized and subsequently populated with endothelial cells (ECs) to generate a vessel with endothelium. To mimic a healthy and diseased connective tissue, hydrogels composed of high collagen or high fibrinogen concentrations were used and fibroblasts incorporated. The vessels were perfused, and stable viability and metabolic co-culture conditions (lactate dehydrogenase, glucose, lactate) were acquired after 3 days for at least 7 days. Angiogenic sprouting and secretion of angiogenic cytokines (bFGF, VEGF, sPECAM-1) were increased in high fibrinogen hydrogels. Perfusion with primary monocytes revealed differentiation into CD68+CD206+ macrophages and immune cell migration across the endothelium into the tissue. In conclusion, this versatile and easy method to pattern hydrogels in MOCs can serve as the basis to build the next generation of vascularized, immunocompetent human MOC models, therefore opening new possibilities in tissue engineering to study health and disease.

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Predictive tools for evaluating environmental chemicals in adipogenesis and obesity: A non-animal approach

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This study focuses on *in silico* and *in vitro* New Approach Methodologies (NAMs) to investigate the potential of environmental chemicals to disrupt adipogenesis, a critical process closely associated with obesity, lipid metabolism, weight gain, glucose/insulin signaling, and other metabolic mechanisms. Understanding adipogenesis is fundamental to uncovering the mechanisms related to overweight and obesity, conditions affecting 2.5 billion adults globally according to the World Health Organization (WHO) in 2022, continuing to rise. These conditions are associated with severe health outcomes, including hypertension, diabetes, and cardiovascular diseases. Considered a multifactorial condition influenced by genetic predispositions, lifestyle habits, and environmental exposures. Referred to as obesogens, some chemicals affect metabolic endpoints, disrupting lipid metabolism and potentially inducing adipocyte hyperplasia and hypertrophy, commonly encountered in daily life, such as in cosmetics, personal care products, and food. To identify new candidates, we curated nearly 200 compounds associated with overweight, obesity, and Non-Alcoholic Fatty Liver Disease from the Comparative Toxicogenomics Database. Two NAMs were applied: (1) an *in silico* model with molecular docking (MD) approach to evaluate compound-protein interactions, and (2) an *in vitro* model using human adipose-derived mesenchymal stromal cells (hADSCs) to investigate potential effects during adipocyte differentiation in the presence of selected chemicals from the MD analysis. These cells were chosen for their adipogenesis capacity, as well as their relevance to human biology. For the *in silico* analysis, we performed molecular docking and virtual screening using AutoDock Vina. Target proteins selected for this analysis were CDK4 and IGF1R (hADSC proliferation), RXR α (pre-adipocyte commitment), PPAR γ (adipocyte differentiation), and FABP4 (lipid uptake). Binding affinities of -6.5 kcal/mol or lower are considered significant, following widely accepted thresholds in MD studies. Among the 70 compounds screened, 60% displayed significant affinities, with 52% exhibiting interactions with four of the five target proteins. Key findings included Bifenthrin (-11.4 kcal/mol with RXR α), Benzo(a)pyrene (-10 kcal/mol with four proteins), Cyhalothrin (-10.8 kcal/mol with RXR α and -8 kcal/mol with PPAR γ , FABP4, and IGF1R), and PFAS (-8 kcal/mol with four proteins). Subsequent analysis revealed the frequent association of these chemicals with food-related applications, such as pesticides, additives, and non-stick agents, among other uses. Following these findings, *in vitro* adipogenesis assays will be conducted using hADSCs in the presence of selected chemicals. Will

be evaluated through phenotypic and gene expression analyses to provide further insights into the biological effects related to the *in silico* predictions. This study reinforces the critical need to develop and refine predictive tools for evaluating not only the biological impacts of environmental chemicals but also for advancing fields such as drug discovery and repurposing. Rising obesity rates and widespread chemical exposure demand such tools to address these global health challenges, supporting regulatory decisions and promoting the protection of human and animal health.

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Facilitating access to micro-physiological systems: Experiences of the establishment of the first of its kind MPS core facility

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Microphysiological systems (MPS), including organoid and Organ-on-Chip technologies, are advanced tools that replicate human physiology and are gaining increasing attention in basic, biomedical, and pharmaceutical research. Despite their advantages over traditional models, widespread adoption of MPS has been hindered by challenges such as limited awareness, the involvement of multiple stakeholders, and the interdisciplinary expertise required to operate these systems. While most research institutions provide well-established infrastructure and trained personnel for animal experiments, comparable institutional support for MPS is still lacking.

To address this gap, the 3R-Center Tübingen is establishing the first of its kind MPS Core Facility. This facility aims to provide researchers with low-threshold access to MPS, enabling them to integrate these models as alternatives or complements to animal experimentation. The Core Facility offers comprehensive support throughout the entire research process, from initial consultations and feasibility assessments to hands-on training, experimental execution, and data analysis, while also providing access to essential infrastructure.

To validate this approach, three research groups were recruited as pilot users during the facility's initial development phase. In the initial evaluation and planning phase, feasibility assessments were conducted, followed by platform and cell source selection as well as consultation on readout strategies. Each project targeted a distinct organ system using established (commercially available) Organ-on-Chip models. During



the implementation phase, non-expert users were trained on the respective platforms and supported in model establishment and experimental execution.

Beyond direct support, the facility empowers researchers to independently implement MPS in their own laboratories, ensuring sustainable long-term adoption and increasing awareness through word-of-mouth within research communities where these technologies are still underutilized. Additionally, the MPS Core Facility serves as a blueprint for similar initiatives, further expanding access to alternative and complementary methods. By lowering entry barriers and promoting human-relevant biomedical research, the facility contributes significantly to the 3R principles, driving progress in both animal-free testing strategies and scientific innovation.

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The Amsterdam example of empowering education for the transition towards animal-free innovations

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The transition towards animal-free models in biomedical science is a pressing need, driven by advancements in technology, ethical considerations, and the demand for human models. However, one of the major barriers to advancing this transition is the lack of widespread, science-based awareness and knowledge about current opportunities and challenges among students and professionals. Education plays a

pivotal role in addressing these gaps, equipping the next generation of scientists with the tools to innovate and improve animal-free methodologies.

As the Education Workgroup of Amsterdam TPI (Transition Program for Innovation without the use of animals), we are committed to facilitating this transition. Proudly supported by the Dutch national TPI initiative and Amsterdam 4R (Reduction, Replacement, Refinement, Reproducibility), our mission is to empower education to advance awareness, knowledge, and skills for the transition towards animal-free innovations.

Our approach includes:

1. Mapping existing education: By identifying current Amsterdam-based courses and training programs related to animal-free innovations, we provide students with a clear overview of where they can access relevant education.
2. Identifying gaps: Through inquiries and surveys, we gain insight into where education on this topic is still missing. This allows us to encourage educators, coordinators, and decision-makers to implement more programs and content in their curricula.
3. Developing targeted courses: We are designing a specialized course for PhD students, equipping these future scientists with advanced knowledge and practical skills to drive the transition.

Our overarching goals include integrating education on the transition towards animal-free research into all (bio)medical curricula across Amsterdam universities and institutes, providing education on the transition towards animal-free models for research professionals, and establishing ourselves as a knowledge hub for education on the transition towards animal-free innovations.

In conclusion, education is a cornerstone of the transition towards animal-free innovations. By creating a robust foundation of knowledge and fostering collaboration, the Amsterdam-based workgroup on education for the transition towards animal-free research aims to inspire and prepare the next generation of scientists to lead the way in animal-free innovations, thereby enhancing science and ultimately improving human health.

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Study of the difficulties encountered by Brazilian laboratories in implementing alternative methods to animal use: Assessment of ocular irritation and the bovine corneal opacity and permeability (BCOP) test

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Introduction: Brazil ranks fourth in the consumption of personal care, perfumery, and cosmetic products, generating approximately \$22.9 billion annually. With the increasing availability of these products and the impact of new generations on the market, Ensuring the safety of products that may cause ocular irritation is crucial, as their use represents a relevant public health concern, especially considering the risks of adverse reactions. The Bovine Corneal Opacity and Permeability (BCOP) test is an accepted alternative method for ocular irritation assessment in regulatory contexts.

Objective: This study aimed to identify the main challenges and technical limitations faced by professionals when implementing alternative methods, particularly for ocular irritation assessment.

Method: A qualitative research approach was employed using questionnaires (n = 22 respondents) and interviews (n = 7), followed by discourse analysis.

Results: Findings reveal that 71% of alternative method practitioners encounter significant difficulties, primarily due to high validation/implementation costs (62%), driven by consumable expenses. Limited access to training (62%) and importation of reagents and equipment (43%) were also noted. Key BCOP limitations include restricted access to bovine eyeballs (50%), less precise risk classification (43%), and substantial eyeball waste (29%) due to damage.

Interviews supported these conclusions, highlighting challenges such as travel to slaughterhouses, insufficient technical details in the OECD Guide, the need for additional support materials, and a lack of experienced professionals. Participants noted difficulties with the availability of eyeballs, often due to incineration requirements or concerns over contamination. Waste from damaged eyeballs was another issue, as was the complexity of classifying irritants. Additionally, travel time and logistical challenges impacted the efficiency of tests, and regulatory constraints on method modifications further complicated implementation. The lack of in-depth training and the high costs of imported materials also hindered progress.

These results underscore the need for expanded training opportunities for test implementation. The high cost of imported materials indicates greater validation expenses in Brazil than in other countries.

Conclusions: The survey identified key challenges faced by Brazilian laboratories in adopting alternative methods for eye irritation testing, particularly BCOP. These include high costs, limited training, and difficulties accessing necessary materials. Overcoming these barriers requires open innovation, fostering collaboration among the Triple Helix (Companies, Scientific and Technological Institutions, and Government). Public policies supporting input imports, funding, and strengthening partnerships are essential to advancing ethical, sustainable toxicological practices.

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No more canaries in coal mines: A systematic review of environmental health monitoring, an evidence-based complete replacement

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Although institutions have traditionally used live soiled bedding sentinel (SBS) mice for rodent health surveillance, increasing evidence demonstrates that Environmental Health Monitoring (EHM) is an effective, evidence-based, cost-reducing replacement. Still, as demonstrated in benchmarking surveys, some institutions have not replaced live sentinels with EHM. They sometimes cite lack of scientific evidence as their reasoning. Therefore, to establish its evidence-base, our team performed a systematic literature review to identify, summarize, and evaluate the efficacy of EHM as compared to SBS.

After systematically searching for and evaluating articles published prior to October 15, 2023, we included 42 peer-reviewed publications in the analysis. Although the design of the articles varied, they included evaluations of exhaust dust testing (n = 27), sentinel-free soiled bedding (n = 8), and direct colony sampling (n = 24). Both quantitative and qualitative analysis showed that all types of EHM were superior to soiled bedding sentinels at detecting pathogens, regardless of pathogen type.

This project demonstrates there is a strong evidence-base for replacing SBS with EHM. Furthermore, in conjunction with this work, the 3Rs Collaborative's EHM initiative has created an extensive resource hub on this topic including lists of institutions that have switched, downloadable SOPs and slide decks, advice on how to switch, a mentorship program, cost analysis, and more. We therefore provide both the evidence for and resources to replace sentinel mice with EHM, which can be used as a case study for how replacement technologies can effectively be integrated in the field.

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Engaging high school students in the 3Rs principles: An educational initiative on animal testing alternatives at the faculty of pharmacy and food sciences of the university of Barcelona

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The University of Barcelona conducts outreach activities for high school students to facilitate their transition to university education, with each faculty offering distinct programs. As professors at the Faculty of Pharmacy and Food Sciences, we have developed an educational initiative titled "Ethics and Research: Alternatives to Animal Experimentation in Pharmaceutical Sciences." This activity aims to provide high school students with an introductory perspective on animal experimentation and the 3Rs concept: Replacement, Reduction, and Refinement. The program is structured as a single-day event comprising three seminars covering: The history of animal experimentation and the evolution of the 3Rs principle. Ethical considerations in animal experimentation. The international legislative landscape regarding the prohibition of animal testing in cosmetics. Alternative methodologies including *in silico* approaches, *in vitro* testing, organ-on-chip technology, organoids, three-dimensional models, and other emerging techniques. Additionally, students are introduced to our faculty laboratories' ongoing research in developing alternative methods. The initiative has garnered significant interest, necessitating its expansion to two separate sessions to accommodate all participating institutions. Four different secondary schools from Barcelona and surrounding area have participated. The demonstrated success of this initiative warrants its continuation in subsequent academic years, facilitating the ongoing exposure of high school students and their teachers to the 3Rs framework and raising awareness about the diverse array of available non-animal testing methodologies. Such educational outreach programs play a vital role in cultivating the next generation of ethically conscious researchers and advancing the implementation of alternative testing methods.

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Molecular imaging for refining pain localization and severity assessment in cisplatin-treated mice

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Introduction: Long-term pain can occur as a side effect of chemotherapeutics, known for their cardiac, hepatic, and neurotoxicity. Especially in preclinical models, where animals cannot verbalize or even hide pain, its diagnosis and localization remain a challenge. Traditional behavioral tests are often insufficient for indicating the type of underlying pain, such as visceral or neuropathic pain, underscoring the need for more advanced diagnostic tools. To address this, our study utilizes PET/SPECT/CT imaging to improve pain localization and severity assessment by quantifying $\sigma 1$ receptor expression and detecting apoptosis in cisplatin treated mice. This approach aims to enhance welfare monitoring and refine severity classification in preclinical research.

Materials and methods: Cisplatin-induced pain was assessed in 20 female Balb/c mice, with 5 additional control mice receiving NaCl injections. Cisplatin (2.3 mg/kg) or NaCl (max. 10 mL/kg) was administered intraperitoneally in two 5-day cycles, with a 5-day break between cycles. PET/SPECT/CT imaging was performed on days 4, 9, 14, and 19 post-treatment, using the $\sigma 1$ receptor-targeting radiotracer [18F]fluspidine and the apoptosis radiotracer [99mTc]Tc-Duramycin. Pain and welfare parameters were also monitored via clinical scoresheets, the Open Field Test, the Mouse Grimace Scale, the Von Frey Test, fecal corticosterone metabolites, and hemograms. Tissue samples were collected for histological analysis.

Results and discussion: Cisplatin-treated animals experienced up to 10% body weight loss and elevated score sheet values during treatment cycles, with partial recovery during treatment breaks, likely caused by nausea. Mechanical sensitivity (Von Frey Test) increased following cisplatin treatment, indicating an elevated pain response without distinguishing between visceral and neuropathic pain. PET/CT imaging revealed increased $\sigma 1$ R expression in the abdominal region, particularly in the intestinal wall, after the first cisplatin cycle, indicating a visceral pain component, potentially associated with gastrointestinal toxicity. This hypothesis was further supported by decreased $\sigma 1$ R expression during treatment breaks (d9, d19), which correlated with partial recovery in the Von Frey Test and with the activity profile (Open Field Test). Additionally, SPECT/CT imaging detected hepatic apoptosis after cisplatin treatment. Hemograms showed macrocytic anemia as a side effect. Ongoing analyses aim to validate these findings.

Conclusion: These preliminary results demonstrate the potential of our PET/SPECT/CT imaging approach to sensitively localize chemotherapy-induced pain and tissue damage, offering valuable diagnostic insights for preclinical studies. While further analysis is required, this



radiotracer combination could significantly enhance pain monitoring and refine welfare assessments in future research and may serve as a reference to established severity tests.

Relevance to animal welfare: This imaging approach enables objective pain assessment, supporting efforts to refine experimental procedures, reduce suffering, and enhance the validity of preclinical pain models.

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A microphysiological model of human foreign body response on a chip

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Implantable medical devices, biosensors and drug delivery systems are integral parts of modern medicine. However, adverse immune responses (collectively known as foreign body response (FBR)), leading to chronic inflammation and fibrosis, are major barriers to clinical success of implantable biomaterials. This has resulted in a significant interest in developing new biomaterials with enhanced immune compatibility. However, biomaterials discovery workflow relies heavily on animal models from murine models to larger animals including non-human primates with significant 3Rs implications. There are also fundamental differences between human and animal models' immune system limiting their biological relevance. Thus, there is a clear need for human based platforms to study FBR to biomaterials.

In this project we describe how human induced pluripotent stem cells (hiPSCs) can be used to develop an *in vitro* model of FBR representing key events involved in recognition of foreign materials and initiation of inflammatory responses against them leading to fibrosis. Macrophages (key immune cells that orchestrate the FBR) generated from hiPSCs showed comparable phenotypical and functional properties to their peripheral blood monocyte-derived counterparts. Autologous fibroblasts and endothelial cells representing tissue/stromal components of the FBR were also generated from hiPSCs and co-cultures of immune and non-immune cells were established under static and flow conditions using a resealable microfluidic device to enable long-term (up to 4 weeks) maintenance of these cells. This platform can be used to investigate inflammatory responses following the exposure of macrophages to exemplar biomaterials as well as the downstream effect on fibroblasts as surrogate for connective tissue responses. Ongoing work is focused on further optimization of culture conditions including cell ratios and inclusion of autologous adaptive immune cells followed by comparing the performance of the model against existing data from animal models.

We believe once fully optimized this platform will enable us to screen the FBR towards biomedical materials in a personalized manner at low cost, higher throughput and rapid timelines not possible before with any other existing *in vivo* or *in vitro* models.

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Fearless and confident – Injections and blood sampling in rodents

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In terms of refinement, we constantly aim to improve our handling of mice and rats. One of the most challenging situations is fixation of animals for injections and blood sampling. The compliance of animals to hold still while being injected is not given, on the contrary, there is a need of fixation for at least a short time to be able to inject safely and correctly. It is even more important for blood draws because this takes longer. Unfortunately, fixation causes major stress in rodents. On top of that, a lot of (unexperienced) researchers feel insecure to properly fixate an animal, especially rats. That transfers to the animals and increases their fear and therefore stress. Here we show how using an injection tunnel can reduce stress in animals and researchers.

At our institute we invented an injection tunnel for rodents. We placed a vetbed in a very short plastic tunnel to create a dark and cozy place for the animal. They walk in voluntarily and make themselves at home. The researcher only needs to hold onto the tail and can quickly inject intraperitoneally. Even drawing blood from the tail is possible without any further fixation. We could observe significant differences in grimace scale and social behavior. Both clearly showed less stress and reduced signs of pain with using the injection tunnel. However, there was no significant difference of catecholamine levels in animals where the injection tunnel was applied versus animals which were fixed with a common restrainer. Furthermore, all researchers agreed on decreased hesitation in approaching and handling the animals.

We conclude that stress can be minimized for animals and researchers likewise if the necessity of firm fixation is omitted. Both animals and people approach the injection tunnel open and fearlessly. The lack of differences in catecholamine levels might be explained with the fact that all our animals are trained before the experiment starts to lose fear of contact with humans. Besides, they all are allowed to go to a playpen at least twice a week. Both lead to trust and confidence and reduce stress levels noticeably. Therefore, our study might not reflect stress reduction concerning catecholamine levels as this might be the case with personnel just starting animal work. Nevertheless, stress reduction is evident in all animals that were injected or had blood drawn with these tunnels. This is an easy and cheap way to improve health and wellbeing of rodents and researchers alike.

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Magnetically guided biofabrication of 3D constructs

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Introduction and objective: Three-dimensional (3D) cell cultures, such as spheroids, are widely used in Tissue Engineering to reproduce *in vivo* cellular environments and to help the formation of complex constructs for tissue regeneration applications. Superparamagnetic iron oxide nanoparticles (FeO NPs) are an interesting alternative material to create such complex structures due to their low cytotoxicity and magnetization, which depends on an external magnetic field, without remanence. This project aims to biofabricate constructs using tissue assembly loading FeO NPs to spheroids for tissue regeneration applications as well as serving as an improved *in vitro* platform for pharmacological testing and disease simulation.

Experimental procedure: Commercial FeO NPs (code: 747319, Sima Aldrich) functionalized with polyethylene glycol (to guarantee material stability and minimize cellular uptake) are being used to guide the fusion of the spheroids. Mesenchymal stem cells spheroids from adipose tissue (ASCs, Lonza) are exposed to different FeO NP concentrations (2, 4, 10, 20, 40, 80, and 100 µg / mL) and later to an external magnetic field. CellTiter-Glo Luminescent Cell Viability Assay 3D is performed to determine the FeO NPs cytotoxicity. Transmission Electron Microscopy can be observed to confirm NP internalization and optical microscopy demonstrates spheroids fusions. The spheroids will also be induced into the osteogenic pathway and exposed to nanoparticles at different time points to analyze the influence of cellular differentiation on the entry of the biomaterial into the system. Finally, histological analysis will examine mineralization before, during, and after bone induction.

Results and discussion: The spheroids were exposed to FeO NPs mentioned concentrations for seven days and no significant variations in sphericity and spheroid formation were shown.

Conclusion: Our preliminary results indicate that nanoparticles probably do not interfere in the spheroid formation, allowing the elaboration of complex constructs for applications in tissue regeneration in a simplified, scalable, and reproducible method as well as serving as a more complex platform for pharmacological testing and disease simulation.

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Development of a cartridge bioreactor for parallelized cultivation and stimulation of a complex fracture healing model

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Musculoskeletal diseases represent a major global health challenge, with their incidence rising due to an aging population. Developing novel therapeutic strategies that progress from preclinical studies to clinical application is essential for effective patient care. Preclinical research relies heavily on small and large animal models because robust *in vitro* systems remain limited.

To address this gap, we developed a preclinical three-dimensional (3D) *in vitro* model that recapitulates the initial phase of fracture healing despite being operated under non-perfused and unloaded conditions. This model is established by co-cultivating fracture hematoma (FH) models with artificial bone constructs derived from self-assembled mesenchymal stromal cells over 48 hours but not for longer. It demonstrates significant overlaps with *in vitro* and *ex/in vivo* models.

Nevertheless, our current model was limited by its static cultivation conditions. Furthermore, mechanical loading – known to influence the musculoskeletal system – has not yet been incorporated. Here, we introduce our bioreactor platform, designed to overcome the aforementioned limitations.

First, our bioreactor platform applies standardized, continuously monitored perfusion to extend the FH cultivation period to 5 days under controlled conditions. Additionally, we developed a new insert-based cultivation chamber to incubate four models in parallel. Using this approach, we could successfully mimic the transition from the initial inflammatory phase of fracture healing to the resolving anti-inflammatory phase by showing the decreased expression of IL6 and IL8 and the enhanced expression of IL4 after 5 days.

Secondly, the platform enables the generation of standardized bone models through defined biomechanical loading. To achieve this, we integrated a robust click-on pressure system that utilizes a pneumatic valve to apply mechanical load. This approach yielded functional bone models, as evidenced by pronounced expression of osteogenic markers including alkaline phosphatase, collagen I, collagen VI, osteopontin, and osteocalcin. Additionally, ongoing mineralization was confirmed by µCT analysis.

The multimodal bioreactor proposed herein enables (i) the simultaneous cultivation of four *in vitro* 3D models under perfused conditions for extended periods, thereby more closely approximating *in vivo* environments. This capability facilitates the study of the transition in fracture healing from the inflammatory phase to the anti-inflammatory phase, and (ii) the application of mechanical loading conditions that influence the fracture healing process. Ultimately, our goal is to evaluate various biomaterials and therapeutic strategies under precisely defined loading and environmental parameters.

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Generation of a human 3D bone model to mimic glucocorticoid-induced osteoporosis *in vitro*

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Osteoporosis is a widespread bone disease characterized by low bone mass, bone fragility and general changes in bone architecture. This disease can lead to bone fractures, pain and ultimately to reduced mobility and thus to a reduced quality of life. Glucocorticoid-induced osteoporosis (GIOP) is the most common form of secondary osteoporosis. This is of particular importance in rheumatology, as these drugs are very often used to treat patients with inflammatory rheumatic and musculoskeletal diseases.

Our main objective was to establish and thoroughly characterize a human-based *in vitro* bone model consisting of bone-forming osteoblasts and bone-resorbing osteoclasts embedded in β -tricalcium phosphate (β -TCP). This model was treated with the glucocorticoid methylprednisolone to induce GIOP. Finally, we evaluated the restorative potential of two commercially available drugs, denosumab and alendronate. In the future, our model is intended to become a general testing tool for drugs against osteoporosis.

Our GIOP model includes both bone-forming osteoblasts and bone-resorbing osteoclasts to enable bone remodeling. We established an osteoclast differentiation protocol using low-attachment plates and cultured the cells for 21 days in α MEM supplemented with 5% human AB serum, 2 mmol L-glutamine, 25 ng/mL macrophage colony-stimulating factor (M-CSF), and 50 ng/mL receptor Activator of NF- κ B Ligand (RANKL). Mesenchymal stromal cells (MSCs) were differentiated on β -TCP to create the framework for the structure of the bone model. Osteoclasts were then added, and the models were treated with methylprednisolone, which induces osteoporosis, and either denosumab or alendronate to test the drugs in a proof-of-principle study. Bone-forming and resorbing activity was monitored using state-of-the-art μ Ct technology, analysis of the supernatant for characteristic proteins and immunohistology.

Multinuclearity, typical β -actin ring formation, cellular activity by tartrate-resistant acid phosphatase (TRAP) staining and functionality in resorption tests proved the functionality of the osteoclasts. Our protocol allowed us to passage these cells without cell loss or loss of function. To establish the untreated bone model, we seeded osteoclasts onto a pre-seeded β -TCP construct (3 weeks) and co-cultured for an additional 7 days. Using μ Ct, we were able to demonstrate homeostasis between bone formation and resorption (functioning osteoblasts and osteoclasts on the model). Furthermore, we analyzed the supernatant and demonstrated a clear secretion of RANKL, osteoprotegerin (OPG), matrix Metalloproteinase-9 (MMP-9) as well as free phosphate and calcium. These results confirmed the functionality of both osteoclasts and MSC-derived osteoblasts in our 3D model. We then transferred our untreated model to the osteoporosis-simulating model by treating it with methylprednisolone and found a disruption of bone homeostasis in favor of osteoclast activity. These effects were partially reversed by the therapeutic agents.

Ultimately, we will obtain an *in vitro* 3D co-culture of osteoblasts and osteoclasts that simulates human bone and is able to mimic important

aspects of GIOP *in vitro* after treatment with glucocorticoids, which can be used for preclinical *in vitro* experiments to investigate available drugs against osteoporosis.

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Impact of the alternative methods implementation in a contract research organization in Brazil

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The registration process for agrochemicals is regulated by agencies such as the Ministry of Agriculture, Livestock and Supply (MAPA), the National Health Surveillance Agency (ANVISA) and the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) in Brazil. This process involves conducting extensive studies to assess the efficacy, safety, and environmental impact of products. Significant progress has been made in replacement of the regulatory *in vivo* test. Several alternative methods (NAMs) are recognized by the OECD to classify chemicals according to the UN GHS, besides the Brazilian legislation has recognized these methods since July 2019 (RDC 294, Anvisa). The Contract Research Organization (CRO) is responsible for managing and performing the entire process of clinical and preclinical trials for products regulations, so it must be committed to the use of the validated guides, as well as to the updated laws. Therefore, the implementation of NAMs is a key point for CROs. In this sense, our objective was to verify the impact of the NAMs after 5 years (2019-2024) of their implementation in our CRO. The main impact was the significant decrease in the number of rabbits and mice, such a reduction was gradual during the 5 years, especially after implementation of OECD 439, 431, 487 and 492B, for example. Additionally, methods to evaluate endpoints such as skin and eye irritation/corrosion use human reconstructed tissue, therefore they can provide more relevant and accurate data for assessing the potential toxicity of agrochemicals. These details are aligned with the principles of the 3Rs (Replacement, Reduction, Refinement), which aim to replace animal testing, reduce the number of animals used, and refine methods to minimize suffering. Some challenges were encountered during these 5 years: NAMs require thorough validation and comparison with traditional animal-based data. It is extremely important to establish the reliability and predictability of these methods, especially for formulated agrochemicals. Overall, the impact of implementing NAMs in the regulation of agrochemicals is largely positive, particularly in terms of animal welfare, efficiency, and the potential for better environmental and safety outcomes. However, the challenges of validation, stand-



ardization, and regulatory adaptation remain key considerations for our CRO. The continued development and adoption of NAMs will likely play a crucial role in influencing the future of agrochemical regulation.

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Time-to-toxicity test to eye evaluation for pesticides: Advantages and limitations

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Pesticides are widely used and indispensable in agriculture production. However, human exposure to them may cause adverse health outcomes. Therefore, it is extremely important to evaluate the eye risks of these substances. Significant progress has been made in the replacement of the regulatory *in vivo* Draize rabbit eye test. Several alternative methods are recognized by the OECD to classify chemicals according to the UN GHS, but any one was not able stand-alone to predict eye hazard potential until 2022. The OECD 492B guideline describes the Time-to-Toxicity test (TTT) using a commercially available reconstructed Human Cornea-like Epithelium (HCE), which mimics the histological, morphological, biochemical and physiological properties of the *in vivo* human corneal epithelium and is capable to classify the three main categories of UN GHS. Most pesticides are agrochemical formulations composed of a complex mixture of substances, usually the Contract Research Organization (CRO) knows only the active(s) ingredient(s), which shows us the need to investigate the entire compound. Our aim was to conduct a retrospective analysis of *in vitro* assays compared to *in vivo* GHS classification, and finally to conclude the advantages of OECD 492B and their limitations to agrochemical formulations. Thereby, 198 studies conducted over the period of 2 years (July 2023 to December 2024) were verified. OECD 492B was able to classify every pesticide independently if it is liquid, viscous or solid, according to UN GHS. However, there is a limitation to differentiating categories 2A and 2B, and some countries still use them, and unfortunately *in vivo* tests were necessary. We found some discrepant results related to literature, which suggest the components influence or different test system were used. Therefore, our results showed the importance of knowing all the formulation and how TTT is extremely useful to address eye damage. Consequently, OECD 492B has the capability to discriminate against the three categories of UN GHS and could be considered a total replacement for classify pesticides. Therefore, the method offers several regulatory advantages,

such as reducing animal testing, ensuring compliance with international standards, and providing more efficient, cost-effective testing methods. It aligns with global regulatory requirements and can help expedite market access for safe products.

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Application of a combined strategy of *in vitro* assays, QSAR, and molecular dynamics to assess the sensitizing potential of antioxidants

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Identifying raw materials with potential of dermal sensitizing is essential for ensuring consumer safety. However, evaluating individual components in isolation may not always provide a complete risk assessment, as interactions within complex formulations can either amplify or mitigate the sensitization potential of specific ingredients. While several standardized approaches exist for chemical risk characterization – including test batteries and tiered assessment strategies based on historical data – access to these technologies remains a challenge in many Latin American countries, often limiting their feasibility due to cost and availability constraints.

Observations of the variable behavior of common antioxidants used in the cosmetic industry when incorporated into different formulations highlighted the need for a more comprehensive characterization. To address this, we employed a combined strategy that integrated *in silico* predictions using the VEGA Hub and CASE platforms with experimental *in vitro* assays, specifically the Direct Peptide Reactivity Assay (DPRA) and the KeratinoSens assay. Discrepancies between *in silico* predictions and *in vitro* results prompted further investigation through molecular modeling and dynamic simulations. This additional analysis allowed us to explore key molecular properties, including electronic charge distribution, solvent-dependent bond formation, and the potential for covalent interactions with epidermal proteins, to better understand the observed inconsistencies.

By integrating data from multiple sources, we identified that two out of the six evaluated molecules exhibited potential dermal sensitization properties. Additionally, we uncovered molecular characteristics that may explain the variability in the observed results. Ultimately, our findings highlight the importance of combining computational and experimental approaches, demonstrating that a well-integrated methodology enhances the accuracy and relevance of sensitization predictions within specific formulation contexts.

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Toward a physioxic organ-on-chip platform with chemically defined cell culture medium

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Introduction: Within the human body, the median oxygen level range is 3-7.4% and is thus considerably lower than in the ambient atmosphere with approximately 20.9%. While most organ-on-chip platforms do not incorporate this aspect, it may be crucial for physiologically relevant experimental results [1].

Materials and methods: To address this issue, a literature review was performed, the microphysiometric IMOLA system [2] was extended toward adjustable oxygen levels, and cellasys #8 assays [3] were performed to investigate a Caco-2 cellular model. The cellasys #8 switched between chemically defined cell culture medium (CCM) and CCM with fetal bovine serum. The assays were performed under physioxic and ambient oxygen levels.

Results: Noteworthy, the oxygen level in a humidified incubator with 5% CO₂ is 18.6% [4]. Also, to allow a low oxygen level of 5% to settle in a flask with 40 mL liquid medium the flask should be stored for approximately 24 h in a low oxygen atmosphere. The measured extracellular acidification rates as well as impedance measurements indicate that the chemically defined CCM performs equally to the CCM with fetal bovine serum. A reduced metabolic activity as well as an alteration in impedance was observed under physioxic conditions.

Conclusion: Further experiments include the investigation of substances with oxygen dependent activity as well as experiments with cancer models and different oxygen concentrations.

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Study of the ecotoxicity of oral antidiabetic DPP-4 inhibitors using machine learning models

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Pharmaceutical residues are increasingly becoming a significant source of environmental pollution worldwide. These compounds and their (bio) transformation products represent a serious environmental problem, often expressed in terms of acute and chronic toxicity to aquatic organisms. Estimates of the total number of active pharmaceutical ingredients (APIs) in global use range from 3,500 to 10,000; however, the environmental toxicity of only a small fraction (< 10%) of these has been investigated. Diabetes is a chronic disease affecting approximately 422 million people, with estimates of reaching 643 million by 2030. The growing prevalence of diabetes and the consequent increase in the use of oral antidiabetic drugs lead to increased environmental pollution by these medications. DPP-4 inhibitors (iDPP-4: Alogliptin, Saxagliptin, Sitagliptin, and Vildagliptin) are a class of oral medications for the treatment of type 2 diabetes mellitus in adults, and their use has increased in recent years. Currently, QSAR models based on machine learning (ML) are promising tools for predicting different toxicity indicators at low cost, with reduced environmental impact, and minimizing the use of animals in experiments. Thus, the objective of this study was to evaluate the environmental toxicity of iDPP-4 and their metabolites using machine learning models. The biodegradation potential, lipophilicity, and toxicity to algae, crustaceans, and fish were assessed using QSAR models implemented in the ADMET Predictor, VEGA and ECOSAR software. The SMILES notation of the 4 drugs and their metabolites was used as input data for the models. For biodegradation prediction, the ML model developed according to OECD guideline 301 was selected, and the results indicated that all iDPP-4 and their metabolites are non-biodegradable, meaning they have aquatic persistence. In the absence of experimental lipophilicity data, logP was simulated using the AlogP v.1.0.1 model. Lipophilicity analyses indicated that iDPP-4 have logP values below 3, suggesting a low potential for bioaccumulation in fish. Alogliptin and its metabolites showed a high risk for green algae, being categorized as highly toxic with short- and long-term effects. The other drugs and metabolites showed low or no toxicity. It can be concluded that iDPP-4 have a low probability of bioaccumulation, reducing the risk of secondary human intoxication, and low lipophilicity, meaning they are more likely to remain solubilized in the environment rather than absorbed. The high risk posed by Alogliptin and its metabolites highlights the importance of regulatory agencies requiring environmental toxicity information for the approval of drug commercialization.

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Invertebrates as replacement model: Are we a modern Descartes?

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The French philosopher René Descartes (1596-1650) defended the idea that animals were automaton beings, devoid of any kind of feeling. This view was later challenged by Jeremy Bentham (1748-1832), who raised that the central issue was not whether animals could reason or speak, but whether they could suffer. Then the seed of animal sentience was sown. For centuries, legal protections for animals, particularly in scientific research, were largely restricted to vertebrates. However, Directive 2010/63/EU expanded these protections to include cephalopods, acknowledging scientific evidence of their capacity for sentience. Recent studies have further demonstrated that even insects, such as bees, are capable of experiencing pain. In the same way that different vertebrate species demonstrate pain and distress in distinct ways, invertebrates are also expected to express these feelings in a different way than vertebrates. Despite this, the lack of understanding about how invertebrates manifest pain has led to their widespread use as alternatives to vertebrates in scientific research. Therefore, to foster a more ethical approach further studies are needed to demonstrate and elucidate the capacity of invertebrates to experience pain, thereby challenging prevailing assumptions and promoting respect for all animals as sentient beings. The continuation of thinking that invertebrates do not feel pain means ignoring these animals as living beings and returning to Descartes' thought.

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Investigating the role of transient receptor potential (TRP) channels TRPM4 / TRPV4 as regulators of colon cancer microenvironment

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Introduction: The microenvironment of tumor cells is considered an essential regulator of the biological behavior of highly malignant neoplasms. Mucins – a family of highly glycosylated proteins – represent a core component of the microenvironment of the healthy and malignant intestine. Work by myself and others identified ion channels of the TRP family as clinically prognostic markers and effective, drug-targeted therapy targets in colon tumors. It is known from basic scientific work that TRP channels control mucin composition and secretion in the intestine. Little is known about the role of TRP channels in the mucin biology of colon tumors.

Objectives: Investigation of a functional connection between activation of selected TRP channels and mucin homeostasis in colon tumors and healthy intestinal epithelium for the development of new therapies or combination therapies for malignant colon tumors.

Methodology: Samples from patients with colon tumors or colon tumor metastases who were surgically treated were examined using molecular biology. For biomarker validation and targeted functional testing of interventions, corresponding organoid-based disease models such as patient-like non-tumor models were generated. To simulate developmental biological specificities of TRP/ mucin regulation during intestinal development, bio-samples was obtained from human.

Results: In the current status of the project, markers could be characterized according to gene and protein quantities. Our preliminary data indicate a dynamic regulation of TRP and mucin composition during tumorigenesis. Continuation of the work will focus on TRPM4/ TRPV4 as primary targets.

Conclusion: My research project validates intestinal organoid technology with regard to its potential to map mucin biology *in vitro*. TRP ion channel-regulated mucin secretion as a potential option for pharmacologically addressable, targeted modulation of the colon tumor microenvironment could contribute to reducing barriers in the treatment of this disease. Functional tests with the generated disease models as well as with genetically mediated inhibition of TRPM4/ TRPV4 under combination therapy with guideline adjuvant chemotherapy are necessary to test the clinical-therapeutic relevance of our hypothesis.

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While we still have to use it: Making the local lymph node assay (LLNA) less burdensome to the mice and safer, non-radioactive for the lab

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The local lymph node assay (LLNA) is an *in vivo* test for identification of skin-sensitizing substances by measuring radioactive thymidine incorporation into the lymph node (OECD test guideline no. (TG) 429). While several non-animal methods and their combinations as defined approaches have been adopted as OECD guidelines (nos. 442C, D and E and 497), the applicability domain of the defined approaches replacing animal-based skin sensitization tests is currently limited to defined organic test substances. In consequence, for formal reasons, any mixture, formulation, UVCB (unknown or variable composition, complex reaction products or biological materials) or other complex test substance as well as any test substance falling outside the applicability domain of OECD guideline no. 497 for other reasons needs to be assessed by other means, i.e., the LLNA or its variants (OECD TG nos. 442A, B) or even the guinea pig test methods (OECD TG no. 406). Furthermore, the LLNA remains the regulatory required assay in some regions. Therefore, as of today and the near future, animal testing is unfortunately required. While the LLNA is already a 3R-method (namely a refinement with less burden) of the original guinea pig methods, it still requires tail-vein injection of radio-labeled thymidine or intraperitoneal injection of BrdU (OECD TG 429 and 442B, respectively).

Here we propose to add an alternative, non-radioactive, label-free proliferation measurement as a refinement of the existing OECD TG 429. In the proposed LLNA cell count method (LNCC), lymph node cell suspensions are subjected to a cell counting procedure. For the validation, cell-counting was added to the radioactive LLNA, so that we obtained both read-outs from the same studies conducted; the cell counts read-outs in general showed lower variability than the radioactive read-outs. In particular, when determining the borderline range (here from routine studies of a single lab), the variability around the respective cut-off values for positivity for the proliferation endpoint measured by means of cell counts is about 50% lower than when using incorporation of radio-labeled thymidine, respectively. Moreover, the equivalent performance of the LNCC and the concurrently conducted radio-labeled-LLNA was demonstrated assessing the core 18 plus the four optional LLNA performance standards resulting [1]. Intra-laboratory reproducibility was demonstrated based on historical control data of hexyl cinnamic aldehyde [2]. Inter-laboratory reproducibility was demonstrated assessing performance standards in three labs in total [3].

Concerning the GHS subclassification, identical GHS sub-classifications were obtained for 20 of the 22 performance standards and based on 101 routine studies there is a good correlation ($R^2 = 0.742$) between the EC3 value derived in the traditional LLNA and the EC1.5 determined in the cell count method [2]. Using cell counts instead of incorporation of radio-labeled thymidine presents an animal welfare advantage, reduces variability (no tail vein injection) and omits the use of radio-chemicals.

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Bridging the gap: OECD guidance on the use of research data in regulatory science

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Bridging the gap between the increasing availability of research data produced from non-guideline studies and the demand for robust scientific evidence to support regulatory decision-making is a challenge. Publication trends can lead to a rapid increase in the number of studies available in the scientific literature. For example, the number of publications associated with the search term “PFAS toxicity” available in PubMed® has increased by an astonishing 4200% over the past decade, with a notable 175% rise occurring in just 2 years. “Research data” is defined here as any scientific data generated in a research context that could potentially inform hazard, exposure, and/or risk assessments of chemicals. It can be generated by scientists from academia, public and private research institutes, industry, or non-governmental organizations. The “OECD Guidance Document on the Generation, Reporting, and Use of Research Data” provides a comprehensive framework to improve the integration of research data into regulatory assessments across OECD Member countries.

The objective of this OECD Guidance is to raise awareness of the benefits and available resources to improve the uptake of research data for regulatory consideration. The document includes specific recommendations for the different groups involved in the life cycle of research data, from data generation to regulatory use (e.g., researchers, funding bodies, publishers, regulatory assessors, risk managers).

The document is organized into four main sections:

1. Policy Challenges and General Principles: An introduction to data quality, scientific relevance, and reliability.
2. Good Practices: Guidance on aligning research with regulatory needs, adhering to reporting standards, and publishing data in accessible formats.
3. Structured Approaches for Data Evaluation: Tools and methodologies for screening, evaluating, and integrating research data, including systematic review techniques.
4. Stakeholder-Specific Recommendations: Tailored advice for improving data generation, reporting, and regulatory integration.

The document also includes appendices with a detailed list of resources and case studies to support practical implementation of research data applied to regulatory assessments. This presentation will outline the key elements of the Guidance and discuss its potential to transform the use of research data in regulatory science, benefiting OECD Member countries and other stakeholders.

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The use of animals and alternative approaches in research, testing and education in Africa

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A new study by Lush Prize assesses the issue of animal use in research, testing and education across the 54 independent states in Africa, the first study of its kind on the “state of play” on animal research and alternatives across the continent.

The report also explores known educational, public awareness and legislative campaigns to end animal use.

Globally, a significant amount of research on live animals is conducted within academic institutions, and this also appears to be the case in various countries in Africa. A review of published scientific papers shows a higher number of results with regard to both animal research and “alternatives” in Nigeria, Kenya, Egypt and South Africa, reflecting greater numbers of academic establishments and general scientific infrastructure, also reflective of the larger nations of Africa.

A great deal of animal research across the continent is related to animal farming (“livestock” production) and associated diseases. The expected increase in animal agriculture, particularly intensive farming, could lead to more animal use in research. Another common area of animal research relates to plant extracts, despite many being of long human use in traditional medicine or as herbal remedies.

No country in Africa has implemented legislation specifically relating to animal use in scientific research or collates/publishes data on the numbers of animals used. While animal protection organizations exist in virtually all African countries, none work solely on the topic of animal research.

There is encouraging use of non-animal approaches, such as *in-silico* and *in-vitro* methods, but these are often used as preliminary work to animal studies, rather than to fully replace them. Nevertheless, this demonstrates the great potential for further training, outreach and education.

On a positive note, many animal welfare organizations already have a well-established program for educational outreach, with the public, schools and university students (particularly veterinary students), which could provide an opportunity for outreach on animal research replacement.

Various workshops have been held over the past decade in several countries to promote replacement methods and our study highlights a few small organizations educating students on replacing animal research in Tanzania, Ghana and Zimbabwe, as well as past initiatives in Kenya.

Much has been made of how Africa can, and is, developing in a way that “leapfrogs” some of the older, more damaging, technologies used in the West (e.g., the use of decentralized renewable energy generation systems instead of giant fossil-fueled power plants and the use of cell-phones and mobile payment systems rather than landlines and banking networks, and we consider there to be opportunities to promote the use of innovative, more predictive scientific techniques that do not rely on animals that would truly “modernize” research across Africa.

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Publishing negative results as a way of avoiding animal use in vain

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Researchers from various fields face problems when they try to publish negative results. In addition to the fact that many journals still do not accept publishing negative results, most researchers still think that these results represent a failure when trying to demonstrate a hypothesis. As a result, the negative results remain in the drawers of the laboratories. This means that these results are not known and there is duplication of studies that are repeated, are not published and the results remain unknown. This may represent a high number of subjects used in the research, whether human beings or animals. The ethical implications arising from the non-publication of negative results in research are immense, not to mention the financial costs arising from the various studies carried out and not published. Firstly, it should be borne in mind that negative results are also relevant results for the understanding of a phenomenon and secondly, it is necessary to change the culture of researchers and scientific journals that must understand the scientific importance of these results. In view of the above, the publication of negative results in research can contribute greatly to preventing animals from being used unnecessarily, contributing not only to the reduction but also to the replacement of the use of these animals, since the simple search in the literature will already demonstrate that there is no need for the use of animals.

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Will animals be completely replaced in the future?

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The answer is simple: No. Perhaps it is an answer that causes astonishment in many people, especially those who are against the use of animals and researchers who seek to replace animals in research. We know that nowadays it is not yet possible to replace the use of animals in all areas, even though we have reached a level of technological development that allows us to make use of various tools that make it possible, if not to replace animals, to reduce the number used in research. These tools range from cell culture to Integrated Approaches to Testing and Assessment (IATAs), through Adverse Outcome Pathways (AOPs), 3D printing, Micro-Physiological Systems, and New Approach Methodologies (NAMs). However, similar to what happens with humans, the development of products for use in animals, whether veterinary drugs, cosmetics, etc., has the non-clinical (pre-clinical) and clinical phases. In clinical studies, human beings are used to test products that will be used on humans, in the same way, animals are used to test products that will be used on animals. The big difference between these studies is that in the clinical phase the products already have a high level of safety. So, we need to intensify the development of non-animal methods, especially for the non-clinical phase because it is of greater risk since all the effects of what is being tested are not yet known. Likewise, the use of non-animal methods should be intensified in quality control. It is precisely where there is no certainty about the safety of a substance or product that animals should stop being used and that we urgently need to replace their use. In the clinical phase, it is difficult to stop using the target species of the product that is being developed, whatever that species may be.

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Integrating structure-based chemical taxonomies to focus queries of NAMs data

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Characterizing and navigating chemical space is a complex challenge, especially when dealing with large and diverse data sets that encompass a variety of chemical structures. This is particularly true for data generated using new approach methodologies (NAMs), as *in silico* and *in vitro* approaches typically yield larger volumes of information than traditional animal methods. One way to focus chemical space exploration is through tools such as ClassyFire (doi:10.1186/s13321-016-0174-y), an automated, structure-based chemical taxonomy tool. Here, we describe efforts to incorporate ClassyFire chemical taxonomies into the Integrated Chemical Environment (ICE; <https://ice.ntp.niehs.nih.gov/>), an open-access web-based resource for toxicologically relevant data and computational tools. We will also present case studies that illustrate how ClassyFire chemical taxonomies can facilitate chemical space exploration within ICE.

We obtained the full list of chemicals in the U.S. Environmental Protection Agency's (EPA's) Distributed Structure-Searchable Toxicity (DSSTox) chemicals database from EPA's Comptox Chemicals Dashboard in August 2024. The 1,125,991 unique InChIKeys in DSSTox were run through the ClassyFire API, resulting in classifications for 1,125,328 structures. These classifications fell into 3,455 unique groupings, with 10 of the 11 levels of the ClassyFire hierarchy represented. "Organics" was the most abundant kingdom (the highest level of the taxonomy), "Benzenoids" was the most abundant superclass (the second level), and "Benzene and Substituted Derivatives" was the most abundant class (the third level). We then integrated these classifications into ICE via a downloadable file.

To demonstrate the utility of this new feature, we used classifications for chemicals tested in EPA's Tox21 program to focus queries of bioactivity and chemical use data in ICE. There were 8,583 Tox21 chemicals with ClassyFire classifications spanning eight levels of the hierarchy. "Benzenoids" and "Organoheterocyclic" were the most abundant superclasses, with 2,744 and 1,959 chemicals, respectively. A total of 3,215 chemicals were bioactive for at least one assay mapped to "Cellular Stress Response." The most abundant superclass for these active chemicals was "Benzenoids" (1,232 chemicals), and the most active class within Benzenoids was "Benzene and Substituted Derivatives" (918 chemicals). We also ran chemicals classified as "Benzenoids" and "Organoheterocyclic Compounds" through the ICE Chemical Characterization tool to examine potential links between chemical class and product use category. Use data were available for 360 benzenoids and 193 organoheterocyclic compounds. For both chemical groups, "Consumer Goods" was the most abundant use category, followed by "Construction/Maintenance" and "Cleaning."

By linking taxonomy to bioactivity and use, users can explore chemical structures and use cases that potentially contribute or relate to toxicological endpoints. This can help focus chemical selection, aid in selection of alternative chemicals, and identify chemical substructures that are correlated with chemical activity.

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The views expressed above do not necessarily represent the official positions of any federal agency.

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Development of a "me-too" eye hazard identification assay using reconstructed human cornea-like epithelium (MCTT HCE™)

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The safety assessment of serious eye damage and/or eye irritation has traditionally relied on animal testing, as outlined in OECD TG405, adopted in 1981. Chemicals that cause serious eye damage are classified as UN GHS Category (Cat.) 1, while those causing eye irritation are classified as UN GHS Cat. 2. Over the past several decades, many researchers have explored alternative, non-animal methods for eye safety assessment. Recently, the SkinEthic™ HCE Time-to-Toxicity test which includes the Time-to-Toxicity for Liquids (TTL) and Solids (TTS) approaches, was adopted in OECD TG492B. This method effectively classifies chemicals (both substances and mixtures) into those labelling for causing serious eye damage (Cat. 1), and eye irritation (Cat. 2), and not requiring classification (No Cat.). This test has high utility and is in strong demand among GLP facilities in Korea. To meet this demand, we developed an alternative eye hazard identification assay as a "me-too" method to OECD TG492B, utilizing the domestically developed MCTT HCE™ Reconstructed Human Cornea-like Epithelium (RHCE). This *in vitro* test method, MCTT HCE™ Eye Hazard Identification Assay (EHA), can be used to classify chemicals into Cat. 1, Cat. 2, or No Cat. following exposure of RHCE cells across various exposure times and measuring cell viability. The MCTT HCE™ EHA was developed using 13 liquid chemicals (five Cat. 1, four Cat. 2, and four No Cat.) and 18 solid chemicals (six Cat. 1, six Cat. 2, and No Cat.). To enhance the predictive capacity of MCTT HCE™ EHA-Liquid (L) and -Solid (S), the doses and concentrations of the chemicals were optimized during the experiment. For the liquid assay (MCTT HCE™



EHA-L), we tested the chemical in neat and at 20% (80 μ L), or 30% (100 μ L) concentrations. Based on the results, the combination of neat and 30% concentration was determined to be the most suitable. For the solid assay (MCTT HCE™ EHA-S), the chemicals were tested in both 80 mg and 100 mg volume, and 100 mg was determined to be the most suitable. Also, we conducted a test to select appropriate positive controls (PCs) for the MCTT HCE™ EHA. Based on the result, methyl acetate was selected as the PC for MCTT HCE™ EHA-L, aligning with TG492B. For MCTT HCE™ EHA-S, 0.1% Sodium Dodecyl Sulfate (SDS) was chosen as the PC, differing from TG492B. The accuracy of MCTT HCE™ EHA was evaluated by testing 31 chemicals (13 liquids and 18 solids) in one run, yielding a balanced accuracy of 74.20% (31 chemicals tested). Specifically, the assay correctly identified 81.81% of Cat. 1 chemicals (11 tested), 50.0% of Cat. 2 chemicals (10 tested) and 90.0% of No Cat. chemicals (10 tested) compared to the reference *in vivo* data. To be specific, the MCTT HCE™ EHA-L exhibited a balanced accuracy of 92.31% (13 tested), correctly identifying 100.0% of Cat. 1 chemicals (5 tested), 75.0% of Cat. 2 chemicals (4 tested) and 100.0% of No Cat. chemicals (4 tested) compared to the *in vivo* data. In contrast, the MCTT HCE™ EHA-S showed a balanced accuracy of 61.11% (18 chemicals tested), correctly identifying 66.67% of Cat. 1 chemicals (6 tested), 33.33% of Cat. 2 chemicals (6 tested) and 83.33% of No Cat. chemicals (6 tested) compared to the *in vivo* data. These results indicate that MCTT HCE™ EHA-L demonstrates a higher accuracy than MCTT HCE™ EHA-S. Further research is underway to optimize the assay and enhance its performance.

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Optimization of siRNA-based inhibition of influenza A virus in a monoclonal human alveolar epithelial cell line for translational 3D lung model studies

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Current vaccines for influenza virus provide only partial protection due to limitations in specificity and efficacy. Small interfering RNA (siRNA) has emerged as a promising strategy for controlling viral infections. Traditionally, various mammalian models have been used in virus research, but some are difficult to infect and require adapted strains. Moreover, these models fail to fully replicate human lung physiology, leading to poor translatability in predicting human responses. This study aimed to establish conditions for inhibiting influenza A virus (IAV) replication using siRNA in a monoclonal human alveolar epithelial cell line (Arlo), to apply these conditions to a human cell-based 3D lung model.

A siRNA targeting the nucleoprotein of IAV was transfected into Arlo cells using Lipofectamine RNAiMAX. Total virus yield, viral genome copy number, and nucleoprotein expression levels were used to analyze the antiviral activity. To investigate the effect of IAV infection on barrier integrity, Arlo cells were cultured under air-liquid interface (ALI) conditions. The barrier function of airway epithelial cells was analyzed by transepithelial electrical resistance (TEER) measurements and immunostaining with antibodies against the tight junction protein ZO-1.

A multi-step growth curve demonstrated that Arlo cells are susceptible to IAV, reaching a viral titer of $10^{6.5}$ PFU/mL at 48 hours post-infection with MOI of 0.25. Similarly, high viral titers were observed when Arlo cells were cultured under air-liquid interface (ALI) conditions. Treatment with siRNA suppressed viral replication in a dose-dependent manner, reducing viral titer by 2.5 to 3 log units. This inhibition was corroborated by decreases in both viral mRNA and protein levels. The barrier integrity of Arlo cells generated a TEER of $1119 \Omega \cdot \text{cm}^2$. This barrier was disturbed during the course of viral infection, as revealed by immunostaining against ZO-1.

We optimized conditions for studying influenza virus replication inhibition by siRNA in both 2D monolayer culture and ALI conditions. These conditions are essential for investigating the antiviral activity in a multicellular human cell-based 3D lung model. Combining this model with siRNA-based inhibition of the influenza virus may provide more translatable results to human pathophysiology, overcoming the limitations of animal models and addressing associated ethical concerns.

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Internalization and implementation of biofabrication platforms and microfluidic systems for alternative methods by the startup 3DBS in Brazil: Expansion and facilitation of access to NAMs

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Tissue and organ *in vitro* models with higher predictive capacity, which lead to results with lower costs and in less time, are necessary for the development of new formulations in the pharmaceutical, cosmetics and food industries. The culture of human tissues in three-dimensional architectures are promising alternatives for these objectives.

Objective: 3DBS startup presents the internalization of the TissUse microfluidics system in its biofabrication laboratory as a platform for New Methodological Approaches (NAMs) in Brazil. The objective is to demonstrate our solutions bringing the challenges related to the implementation and how this initiative expands access to alternative methods in the country. This expansion occurs both through the provision of specialized services and the offering of solutions that reduce bureaucratic barriers to importing technology. In addition to providing the TissUse microfluidic system, we offer advanced biological models, such as liver spheroids, intestinal barrier, reconstructed epidermis (RHE) and full-thickness skin. In this way, 3DBS not only makes this technology available, but also offers technical support, consultancy and strategic partnerships for the development of new personalized models, meeting the needs of academic and industry research.

Conclusion: With this approach, we strengthen the national infrastructure for alternative methods, reduce bureaucratic barriers to the adoption of new technologies and drive innovation in the sector. Our commitment is to consolidate an accessible and efficient ecosystem for NAMs in Brazil, aligned with international regulatory trends and promoting more ethical and effective alternatives to the use of animals in tests. Report a Brazilian startup's experience in offering such tissues and the MPS form of testing.

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Investigating the hepatotoxicity and potential carcinogenicity of microcystins using a human hepatocyte model with gene and cellular markers

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Microcystins (MCs) are cyanotoxins ubiquitously present in the environment and recognized as contaminants in water and food. The MC group comprises 320 congeners, with limited hazard data for most of the compounds. The limited available data predominantly indicate hepatotoxic effects, while MC-LR, the most well-characterized congener, also exhibits tumor promotion activity. These effects are generally attributed to their ability to inhibit PP2A activity; however, the precise mechanisms of action remain to be fully elucidated. To address data gaps in the hazard assessment of MCs, this study investigated the hepatotoxic mechanisms and potential carcinogenicity of MC-LR, MC-LA, and MC-RR, which differ in cellular uptake, detoxification efficiency, and structural characteristics (minor amino acid position variations). These MCs were tested in the differentiated HepaRG model at non-cytotoxic concentrations (0.3, 1.1, 2.5 μ M), using assays to quantify cell proliferation (cell cycle with PI by flow cytometry), PP2A activity (Ser/Thr Phosphatase assay kit 1, Merck), and the expression of protein-coding genes (RT2 Profiler™ PCR Array Human Molecular Toxicology Pathway-Finder 384HT, QIAGEN) and miRNA (Human Cancer PathwayFinder X-Large Focus (miScript 384HC) array, QIAGEN). All three MCs at 2.5 μ M similarly inhibited PP2A (approximately 0.25-fold inhibition); however, significant proliferative effects were observed only for MC-LR and MC-LA at the two highest test concentrations. The qPCR data (2.5 μ M) further revealed differences among the congeners. Principal component analysis (PCA) demonstrated strong and similar treatment effects induced by MC-LR and MC-LA, whereas MC-RR exhibited effects closer to the control. Regression analysis also indicated a strong correlation between MC-LR and MC-LA ($R^2 = 0.6612$), while MC-RR displayed weaker correlations with MC-LR ($R^2 = 0.3076$) and MC-LA ($R^2 = 0.05901$). Notably, genes exclusively expressed in response to MC-LR and MC-LA, but not MC-RR, were associated with xenobiotic metabolism (CYP2B6, CYP2C19, CYP2C9, CYP3A4, GSTA3, and ACO1), inflammation (IL-6), and oxidative stress (CAT, APOF, APOE, and GPX2). According to Ingenuity Pathway Analysis (IPA), the most affected disease or functional categories for MC-LR and MC-LA included liver inflammation, liver cell/hepatocyte proliferation, liver damage, hepatocellular carcinoma, liver fibrosis, cholestatic alcoholic hepatitis, and intrahepatic cholestasis. For MC-RR, only intrahepatic cholestasis and liver cancer were predicted to be affected and with lower significance than the other two congeners. The miRNA data also indicated a stronger association with carcinogenesis for MC-LA and MC-



LR compared to MC-RR, as reflected by both the number of molecules and p-value. In summary, the overall findings suggest that PP2A inhibition may not be the sole key primary event underlying the hepatotoxicity and carcinogenicity of MCs. Regarding carcinogenesis, one possible mechanism for hepatocellular carcinoma induction involves oxidative stress via the IL-6/JAK2/STAT3 pathway. However, further studies are needed to elucidate these mechanisms more comprehensively.

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The future of 3Rs: The day alternatives will be the routine

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The first philosopher to raise the idea that animals are sentient beings was Jeremy Bentham (1748-1832) when he put it that “the question is not whether animals can speak or whether they can reason: the question is whether animals suffer”. Although little was done during this time until Russell and Burch wrote about Replacement, Reduction and Refinement, in general, some actions started at the end of the 1970s when several movements against the use of animals by the cosmetics industries boosted the development of the so-called alternative methods. Nowadays it is still not possible to replace the use of animals in all areas, however, the number of non-animal methods has grown enormously due to technological development that allows greater knowledge of the mechanism of action generating AOPs (Adverse Outcome Pathways), the emergence of tools such as Micro-Physiological Systems (MPS) and developing new forms of evaluation, such as the New Approach Methodologies (NAMs) that allow us to draw conclusions from the gathering of results from various sources ranging from literature searches to experimental results. This growth in technology will allow, within a few years, that a large part of the experiments on animals can already be replaced, when then, the so-called alternative methods will be the common methods normally used, especially in non-clinical studies and in the development of new products and formulations.

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The history of MAT (monocyte activation test) in Brazil

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The Rabbit Pyrogen Test (RPT) has long been used for the detection of pyrogenic contamination in injectable products. Studies on the etiology of fever led Poole (1988) to describe the possibility of “*in vitro*” release of inflammatory mediators by monocytic cells (MonoMac-6). Wendel and Hartung (1995) then developed a model using human blood that would later become the Monocyte Activation Test (MAT). This method was brought to Brazil through a Germany-Brazil agreement (BMbF and CNPq). Several Master’s dissertations, Doctoral Theses and scientific articles have been produced demonstrating the applicability of MAT in the quality control of serums, vaccines, large volume parenteral and air. BraCVAM recommended to the National Council for the Control of Animal Experimentation (CONCEA) the officialization of the MAT, which occurred in 2019, becoming mandatory in Brazil in 2024. Seminars were held together with the Humane Society International to raise awareness about the use of MAT, as well as to identify difficulties that institutions were encountering to implement the method. BraCVAM also recommended the adoption of the MAT as a monograph of the Brazilian Pharmacopoeia and participated in the Working Group that generated the text that was published in the seventh edition of the Brazilian Pharmacopoeia in 2024.

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***In vitro* injury model using reconstructed human epidermis at different maturation stages**

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The skin, a vital organ that acts as a protective barrier, is vulnerable to injuries such as wounds and burns. While animal models are frequently employed in wound healing research, they present ethical concerns and limitations in predicting human responses. Reconstructed human epidermis (RHE) models, like SkinEthic[®], offer a promising alternative, yet their application to epidermal wound healing studies remains underutilized. This study, therefore, aims to develop a reproducible *in vitro* RHE injury model for the investigation of epidermal healing.

Four injury induction methods were investigated: (1) scratch assay; (2) standardized stamp using a biopsy punch; (3) physical barrier incorporation during RHE production; and (4) mechano-thermal injury simulating a burn.

For the development of a reproducible *in vitro* injury model in RHE, several methods were initially evaluated for the scratch assay, including pipette tips, microcannulas, glass capillaries, scalpels, needles, and biopsy punches of varying sizes and application techniques (mechanical stamp). Histological analyses revealed heterogeneous lesions, epidermal-membrane detachment, or membrane rupture, rendering these methods unsuitable. Microneedling, tested with needles of 0.25mm, 0.5mm, 0.75mm, and 1mm depths, produced standardized lesions within the same tissue macroscopically, but with microscopic inconsistencies between samples. Near-IR laser application did not result in significant lesions.

To create a controlled injury, the introduction of a physical barrier during RHE production was investigated. A 3D-printed circular mold resulted in tissue thinning around the injury. A second, truncated cone-shaped mold was tested, but cell migration into the mold area hindered lesion formation. Finally, the use of a mechano-thermal stamp with a circular tip demonstrated promising results, generating uniform and consistent lesions. Histological analysis revealed epidermal thinning, extracellular matrix disorganization, and necrosis in the injured area immediately after application, with subsequent thickening of the central area after 72 h, suggestive of re-epithelialization.

Therefore, the mechano-thermal stamp model was selected for further investigation. Subsequent research will characterize re-epithelialization in this model, analyzing keratinocyte migration, proliferation, differentiation, and biomarker expression. Molecular analyses, including immunofluorescence, ELISA, and Western blotting, will be performed on injured tissues. Re-epithelialization will be analyzed at different maturation stages and under the influence of potential accelerating factors. This work advances our understanding of epidermal wound healing, contributing to innovative therapeutic strategies, particularly in the field of regenerative beauty by providing a more human-relevant platform for testing and developing novel ingredients and formulations. This improved understanding promotes wider adoption of these *in vitro* methods.

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"Let those involved report": Facts, emotions, and coping strategies for enhancing animal welfare in research

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Animal experiments are a crucial component of biomedical research; however, they are increasingly criticized by both society and the scientific community due to bioethical debates and issues related to translation and reproducibility. These concerns are reflected in increasingly strict legal regulations that mandate higher animal welfare standards worldwide, including rigorous regulatory and approval processes, along with internal and external control mechanisms.

As a result, research institutes are currently facing growing expectations for improved animal welfare in experimental protocols. This shift is driven by efforts to implement refinement measures, reduce animal stress during handling, and enhance the continuous living conditions of animals year-round. These stricter regulations and rising expectations bring both opportunities and challenges. On the one hand, they drive important bioethical advancements and enable the implementation of valuable refinement measures, such as improved housing, training and habituation programs, and enhanced experimental handling methods. On the other hand, they also place greater demands on scientists and institutions, increasing their workload and responsibilities.

Researchers must carefully balance conducting high-quality science with fulfilling ethical responsibilities. This challenge is further complicated by concerns over compromised scientific validity, loss of data standardization, increased workloads and time investment, financial constraints, and psychological pressures. At Charité – Universitätsmedizin Berlin, Germany, one of Europe's largest medical universities, where approximately 50,000 animals are used annually for scientific purposes, researchers actively discuss these complex challenges and how they can be addressed. They are supported by a dedicated 3R Center established in 2018 which serves as an independent hub for collaboration and networking and assists the scientists by providing funding and fostering a supportive workplace culture. In this



conference presentation, we would like to summarize the experiences of our scientists, which underline the importance of problem-solving approaches and the need for promoting bioethical awareness, institutional support, and collaboration. Coping strategies need a collaborative approach between different stakeholders. Funders and journals need to recognize animal welfare for scientific validity, insist on its implementation and increase funding for better animal housing and handling. Supportive frameworks at scientific organizations such as 3R centers are needed to allow the best possible refinement introduction. By addressing unresolved challenges, acknowledging emotional burdens, and confronting unspoken issues, researchers can reshape animal experimentation practices, ensuring both scientific progress and ethical accountability.

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Evaluation of the endocrine activity potential of cosmetic ingredients using *in silico* tools

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There is growing awareness of the potential risks associated with cosmetic ingredients, stressing the need for thorough safety evaluations to protect both human health and the environment. Comprehensive assessments are required, particularly for endocrine-disrupting chemicals (EDCs), exogenous substances that can interfere with the endocrine system and potentially cause adverse effects on development, reproduction, and metabolism. In order to screen cosmetic ingredients,

exhibiting low toxicity, rapid and low-cost methods are increasingly required. *In silico* approaches are mathematical models that estimate the activity of substances based on their chemical structure and are extremely useful when experimental data is scarce. This study employed *in silico* methods to predict the potential endocrine activity of well-characterized cosmetic ingredients, comparing the predictions with existing *in vitro* data. The aim was to validate a strategy that uses well-characterized substances to predict the properties of data-poor chemicals. Therefore, 13 substances under evaluation in the EU Endocrine Disruptor Lists were selected based on their relevance as cosmetic ingredients. Additionally, the availability of *in vitro* data on endocrine disruption was assessed using the US EPA Toxicity Forecaster (ToxCast) database, which provides screening tests results to prioritize and characterize the hazard and risk of millions of chemicals. Data on the binding on the binding affinity of these substances were collected for the following receptors: estrogen (ER), androgen (AR) and thyroid (TR), evaluating their potential to act as either agonists or antagonists. The selected substances were predicted using different *in silico* platforms, such as VEGA, OECD QSAR Toolbox, and TEST. A majority consensus approach was applied to score the predictions of each model, assigning scores 0 and 1 for negative and positive results, respectively. The average results were classified as endocrine activity potential (> 0.5), or non-endocrine activity potential (< 0.5). The *in silico* predictions were compared with *in vitro* ToxCast data. For the *in silico* predictions related to ER receptor binding, 9 out of 13 scores confirmed the *in vitro* results (69%), while for the AR receptor prediction, 8 out of 13 results (62%) confirmed the *in vitro* results. For TR receptors, only 6 out of 13 *in silico* results confirmed the *in vitro* results (46%). Predictions for ER and AR demonstrated an acceptable correlation between *in silico* and *in vitro* data, but for TR, less than 50% of the results were confirmed. This lower is probably due to a lack of information on these receptors and fewer *in silico* models available for this endpoint. The study demonstrated that these models are effective in predicting ligand activity for ER and AR when compared to *in vitro* data and that combining multiple models enhances the chemical coverage and improves the identification of potential endocrine-disrupting compounds. Also, it was shown that predictions are generally consistent with *in vitro* data, particularly for receptors with well-documented data. Given the growing need for New Approach Methodologies (NAMs) in hazard identification for cosmetic ingredients, *in silico* methods represent valuable tools, with possible use for data-poor substances.

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Modeling the disruption of epithelial barrier integrity in inflammatory bowel diseases in 2D and 3D cellular models – Comparison with human data and animal models

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Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a group of chronic conditions that affect the gastrointestinal (GI) tract characterized by aberrant mucosal inflammation and immune dysregulation resulting in loss of the epithelial barrier integrity. In this study we investigated the role of Receptor for Activated C Kinase 1 (RACK1) in inflammatory bowel disease (IBD), with a particular focus on comparing the results obtained in animal models and *in vitro* models.

RACK1 is a scaffold protein involved in multiple cellular processes. In the intestine, RACK1 is involved in epithelial cell adhesion, differentiation and apoptosis, as well as crypt cell proliferation and regeneration. Accordingly, RACK1 downregulation is associated with impaired epithelial barrier integrity in IBD and positively correlates with the expression of GC-induced leucine zipper (GILZ or tsc22d3), an anti-inflammatory protein that play a pivotal role in this context.

From the evidence that in humans RACK1 expression is reduced in IBD-affected tissues, we used animal and cellular models to investigate the mechanisms underlying this defective protein. Animal models

of colitis reproduced the anatomical and functional characteristics of the disease and demonstrated reduced expression of RACK1 in the affected tissue. RACK1 expression was downregulated in animal models of GILZ depletion. We wanted to investigate whether these pathological and histological features could be modeled in 2D and 3D cell co-culture models.

2D co-cultures consisted of differentiated Caco-2 cells co-cultured with PMA-differentiated THP-1 M1 macrophages. The inflammatory environment was induced, by treating the cells with lipopolysaccharide (LPS) and interferon gamma (IFN γ). This inflammatory stimulus increased pro-inflammatory cytokines, such as TNF- α and IL-8, and induced RACK1 downregulation in Caco-2 cells. This was consistent with the observed disruption of epithelial barrier integrity which was also confirmed in RACK1 silenced Caco2 cells. Similar results were obtained in 3D co-culture models, which were constituted by a system that includes a lamina propria-like compartment created using fibroblasts and immune cells on an Alvetex scaffold, with a Caco-2 monolayer on top.

Although these *in vitro* models do not include T cells and may not fully recapitulate the complexity of IBD, they can nevertheless be regarded as valuable tools with which to study the mechanisms and pharmacological aspects of IBD, as our data illustrate. Consistently with patients and in *in vivo* data, RACK1 expression is significantly reduced in our 2D and 3D co-culture models of IBD. This downregulation correlates with decreased binding of RACK1 to Src, leading to a reduction in E-cadherin expression and impaired barrier integrity. The research further demonstrates that RACK1 expression is positively regulated by the c-Rel transcription factor. Additionally, the study finds that GILZ positively regulates RACK1 expression through c-Rel, and the loss of GILZ results in decreased RACK1 expression and impaired barrier function. Notably, dexamethasone treatment restores RACK1 expression via the glucocorticoid receptor (GR).

Overall, the study provides evidence that the RACK1/Src/E-cadherin pathway is involved in epithelial barrier disruption in IBD and may represent a therapeutic target for the development of new treatments.

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Retinoic acid mediated patterning in neural tube organoids: Insights into early neural development and defects

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The development of the central nervous system (CNS) is preceded by the formation of an antecedent structure, the neural tube. Interrupted neural tube formation results in severe CNS malformations termed neural tube defects. Neural tube organoids (NTOs), 3D aggregates of pluripotent stem cells, are induced to differentiate and self-organize to recapitulate early development as well as the structure and function of neural tissue. Organoids are a critical New Approach Methodology that enables the intricate study of human developmental events such as neural tube formation, replacing the need for animal models.

A morphogen understood to play a significant role in early neural development is retinoic acid (RA); while studies have demonstrated its importance in neural tube patterning and neurogenesis, the precise role RA plays in neural tube development remains unclear.

This project aims to generate an *in vitro* model of neural tube development using pluripotent stem cells (PSCs) through which the developmental and morphological roles of RA are investigated via pulse treatments and the formation of signaling centers.

NTOs were produced by aggregation of 500 PSCs, which were then treated with pulses of RA at a concentration of 250 nM from 48 hours to 72 hours and grown up until day 6. To form signaling centers, 100-cell aggregates treated with RA from 40 hours to 48 hours were then combined with untreated 400-cell aggregates; the resulting 500-cell aggregates were then cultured for a further 4 days. Following the 6-day culture period, both groups were assessed for temporal and spatial neural marker expression using ICC and RT-qPCR and were analyzed for axial elongation and size by area.

Results from RT-qPCR showed significantly higher expression of spinal cord, hindbrain, and midbrain markers, demonstrating a posterior neural identity, as well as ventral markers, in RA-treated aggregates compared to no signaling controls and aggregates treated with either BMP4 or Chiron. Additionally, the upregulation of somite markers indicates a balanced trunk-like structure has formed. ICC revealed high expression of SOX2, a neuromesodermal progenitor marker, and the formation of tube-like structures indicated by apical F-actin localization. These results indicate a strong posteriorizing and ventralizing bias of RA in early neural development. Furthermore, the formation of tube-like neural rosettes in RA-treated aggregates suggests a key role in the early stages of neural tube formation.

In conclusion, our findings highlight the importance of *in vitro* NTO models in the study of early neural development and their potential to direct future applications towards animal-free disease modelling and therapeutic applications.

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A non-animal toolbox informed by pulmonary toxicity adverse outcome pathways (AOPs): A next-generation risk assessment (NGRA) approach for human inhalation safety

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The assessment of chemical-induced lung effects has historically been achieved by performing animal testing, which has significant limitations (e.g., biological differences between rodent and human respiratory systems and ethical concerns). This study investigated the feasibility of a new approach methodologies (NAM)-based toolbox using commercial human lung models, MucilAir™-HF and EpiAlveolar™, to provide protective points of departure (PoDs) and bioactivity exposure ratio (BER) estimates. Eleven benchmark chemicals were tested, including inhaled materials and drugs that may cause lung toxicity following systemic exposure, covering 14 human exposure scenarios classified as low or high risk based on historical safety decisions. Directly mapped onto the AOPs relevant for lung toxicity and non-specific bioactivity, different readouts were investigated through a 12-day repeated exposure scenario, including tissue integrity and functionality, cytokine/chemokine secretion, and transcriptomics. For calculation of BERs, the PoDs derived from the substances-induced bioactivity were combined with human exposure estimates that were obtained using multiple path particle dosimetry (MPPD) exposure modelling or literature maximum plasma concentration (C_{max}). For the benchmark chemicals investigated in 14 human exposure scenarios, it was possible to correctly separate their risk classification by using the lowest BER per readout and lung model. Generally, for the low-risk chemical exposure scenarios, the PoDs occurred at higher concentrations than the corresponding human exposure values, whereas for all the high risk chemical exposure scenarios, there was an overlap between the PoDs and lung deposited mass or C_{max}. Our findings provide a proof of concept by establishing the feasibility of a toolbox that combines computational and *in vitro* NAMs to be included within a human inhalation safety NGRA approach.

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Combining molecular and mechanical biomarkers in a preclinical lung model to improve anti-fibrotic treatment strategies

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Rationale: Pulmonary fibrosis (PF) is a progressive lung disease marked by excessive extracellular matrix (ECM) deposition, increased tissue stiffness, and disrupted lung architecture. Currently, no curative treatment exists, and effective therapies remain limited. While animal models are commonly used to study PF, they often fail to fully replicate human disease pathology, limiting their predictive value for therapeutic responses. To address these challenges, our study applies the 3Rs principle – Replace, Reduce, Refine – by using human tissue, enhancing data relevance and providing a superior alternative to traditional models. We employ human precision-cut lung slices (PCLS) as a translational model to explore molecular biomarkers and tissue mechanics, with the goal of identifying predictive biomarkers and evaluating anti-fibrotic drugs.

Methods: Human PCLS were obtained from patients with and without PF and cultured with or without nintedanib or pirfenidone for up to 96 hours. RNA was extracted for next-generation sequencing. Cytokine levels were analyzed via ELISA, while microRNA levels in the culture supernatants were quantified using RT-qPCR. Tissue stiffness was assessed using the MechanoWell[®] system.

Results: After 24 hours of culture, a comparative analysis of non-fibrotic and fibrotic PCLS revealed significant regulation of 3,117 genes, with 1,802 genes upregulated and 1,315 downregulated in PF tissue. Among the upregulated genes, ECM-associated matrix metalloproteinases (MMPs) and collagens were prominently enriched, both of which are critically involved in lung fibrosis progression and tissue stiffening. At the protein level, median concentrations of pro-collagen I alpha 1 (pCol1a1) were 3-fold higher, MMP9 were 43-fold higher, MMP7 were 2-fold higher, and TGF- β were 6-fold higher in PF supernatants compared to non-PF controls. Moreover, miR-21 has been identified as a potential biomarker involved in the fibrogenic activation of pulmonary fibroblasts and lung fibrosis. Soluble miR-21 levels were 4.1-fold higher in PF-PCLS compared to non-PF-PCLS.

Treatment of fibrotic PCLS with nintedanib and pirfenidone significantly reduced parenchymal stiffness to 61% and 64% of control levels, respectively. After 48 hours of culture, nintedanib treatment resulted in a marked reduction of MMP9 to 68%, TGF- β to 79%, and pro-collagen I alpha 1 to 68%, while MMP7 remained unchanged. Pirfenidone also decreased MMP9 by 83%, but it led to a significant reduction in MMP7 by 75%, with no significant effects observed for TGF- β or pCol1a1. Additionally, preliminary data suggested a trend toward miR-21 downregulation to 72% following pirfenidone treatment, whereas nintedanib had no significant effect on miR-21 expression.

Conclusion: Our study combines molecular profiling and mechanical analysis of tissue stiffness in a fibrotic PCLS model, a powerful tool for studying lung fibrosis. This integrative approach advances our understanding of PF pathophysiology and provides a framework for evaluating anti-fibrotic drug candidates. Given the limitations of current therapies, such models are crucial for developing more effective treatments, focusing on both molecular and biomechanical parameters to predict therapeutic outcomes. By using human tissue, this model closely simulates human physiological responses, reinforcing the relevance of our findings while supporting the ethical reduction of animal experimentation in fibrosis research.

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Internal validity in rodent forced swim test studies: A meta-regression on antidepressant effect estimate

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The forced swim test in laboratory rodents (FST) was used for predicting the effects of antidepressants in thousands of publications since its standardization by Porsolt in 1977. The systematic review and meta-analysis (SRMA) are suitable tools to make sense of this extensive literature. SRMA may obtain conclusions on the effects of antidepressant treatments in the FST without any additional animal use and allow for appraisal of research quality, guiding improvements in future studies. Therefore, SRMAs align with 3R principles, contributing to reducing, refining, and replacing animals in research. This SRMA (PROSPERO: CRD42020200604) aims to address the following questions: (1) How good is the internal validity of FST studies? (2) Does internal validity influence the combined effect sizes (CES) of antidepressants in the FST? Search terms (“rodents”, “prototypical antidepressants”, “FST”, or synonyms) retrieved 8,247 references ($k = 5,137$ after deduplication) from EMBASE and PubMed (publication date 1977-2017). Screening by two independent reviewers (the third reviewer resolved discrepancies) included 2,584 relevant studies in a library. A random sample of 200 publications from the library (“randbetween” Excel’s function) were appraised in terms of experimental qualities (adapted CAMARADES checklist) and internal validity (SYRCLE’s RoB tool), which levels (low, high, or unclear risk of bias) were included as moderator in a meta-regression on the CES. One reviewer extracted data, cross-verified by a second, and inconsistencies resolved by a third. Experimental quality assessments revealed that reports omitted key methodological details (e.g., housing conditions, animal phenotype, FST protocols, outcome acquisition) and adherence to guidelines (1% following ARRIVE guidelines, 65% vaguely referenced “laboratory animal guidelines”). All studies exhibited an overall unclear risk of selection and performance bias mainly due to insufficient reporting of blinding and randomization practices. Most studies (99.5-96%) presented group similarity and outcome selection information. High-risk biases were rare. In mice studies, low or unclear risk of bias in allocation concealment (CES reduction: -16.70 or -16.62, $p < 0.0001$) and random housing (CES reduction: -19.98 or -22.12, $p \leq 0.0001$) showed markedly smaller CES as compared to high-risk. Heterogeneity ($I^2 = 80.79\%$) and residual variance ($\tau^2 = 1.83$) persisted, although the moderator explained 11.51% of the variance in the model (intercept: 44.3, $Q = 157.33$, $p < 0.0001$). In rats’ studies, the risk of bias had a weaker and inconclusive influence on the CES (intercept: -3, $Q = 22.73$, $p = 0.012$; $I^2 = 78.69\%$; $R^2 = 3.54\%$). In summary, poor reporting hindered the appraisal of the internal and external validities of studies and jeopardized the accuracy of CES estimates. These findings emphasize the need to improve reporting practices in peer-reviewed publications employing FST, with greater adherence to reporting guidelines to enhance research transparency, reproducibility, and ethical compliance.

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Implementing low stress handling across a global organization: Big improvements in animal welfare through small changes in human behavior

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Tail handling of mice and rats is aversive. It causes stress for the animals, degrades the human-animal bond, and is detrimental to animal welfare. Regardless, tail handling is still pervasive in the research environment globally. Low stress handling (LSH), including both tunnel and body or cup handling, provides a data-backed, welfare-friendly alternative method for handling rodents. LSH results in less anxiety and depressive-like states and improves behavioral and physiological parameters. Given the growing body of evidence, there is an ethical imperative to change the culture around handling rodents. As such, Charles River implemented a global LSH initiative in 2022. An initial, company-wide survey assessing current handling methods in 2022 showed that 92% of sites were using tail handling as a primary handling method for mice, and that 79% of sites were using tail handling to some extent for rats. At that time, the primary perceived challenge to implementing LSH was a concern that it would take more time to perform job functions. With this baseline measurement, a change management strategy was initiated to support successful uptake of LSH. The first step was to identify key stakeholders, sharing the evidence from the literature, and securing high-level support while remaining flexible in the approach to LSH uptake. The second step was to engage employees across cultures and business units and effectively train them on LSH methods. Personnel at various levels of the company were involved in LSH discussions and developing messaging to garner support. Additionally, targeted trainings with tunnel, body, and cup handling were provided. The third step was to implement LSH by using clear communication and a piecemeal roll out plan based on local comfort and confidence in the method. The final step was to evaluate the process by asking for feedback and collecting observations and reflections locally. Local findings were then shared out to help support the global Charles River community and build confidence that change was possible. Through this careful process of change management, as of February 2025, the company had achieved 87% compliance with LSH, with 60 of 69 sites on 3 different continents having transitioned to using LSH for mice and rats. Site-level surveys reported that while LSH initially took more time, once employees were trained on using LSH, there was no increase in time to perform job functions and employees reported more positive interactions with the animals compared to using tail handling.

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Welfare recommendations for harmonized care of laboratory rabbit (*Oryctolagus cuniculus*) across a global organization

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Harmonization of research rabbit care and management is essential to maintain a high standard of welfare and robust and reproducible scientific data. The laboratory rabbit is a common and vital animal model. While they are relatively easy to house, maintain, and even breed to support scientific research, there is significant variability in how this is achieved between research facilities. The Rabbit Welfare Working Group at Charles River was established to address inconsistencies between sites and potential gaps in knowledge and improve harmonization of rabbit care across numerous facilities and different business units. The Rabbit Welfare Working Group was comprised of laboratory animal professionals working with rabbits from a variety of research environments. Members of the working group were primarily veterinarians, but also included animal technical personnel, behavior specialists, technical trainers, operations managers, and study directors. The working group addressed 7 topics: (1) basic behavioral needs and housing considerations, (2) social housing, (3) low stress handling and restraint, (4) routine animal care practices, (5) perioperative care, pain assessment, and humane intervention criteria, (6) euthanasia, and (7) animal welfare assessment. Through discussion and consensus building, the group developed 12 recommendations of best practices for rabbit behavioral needs, housing considerations, social factors, handling and restraint, procedural refinement, recognition of pain and distress, humane interventions, welfare assessment, and euthanasia procedures. The recommendations are used as a tool to monitor sites over time. The rabbit welfare assessment tool also allows for objective review of rabbit management programs, helping sites identify priorities for improvement. The successful implementation of these recommendations across multiple business units, countries, and facilities at a large, global, contract research organization demonstrates that they are practical and beneficial for harmonization of rabbit welfare across the scientific community.

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Advancing ocular toxicity assessment: A limbal explant model for evaluating corneal wound healing

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Assessing ocular safety is essential for global product regulation, but traditional animal-based assay, the Draize rabbit test, face ethical and scientific limitations. The Bovine Corneal Opacity and Permeability (BCOP) assay is widely used to identify severe irritants (GHS Category 1) and non-irritants (GHS No category). However, it fails in evaluating moderate, reversible effects (GHS Category 2) and does not assess corneal wound healing which is required for the UN-GHS classification of middle categories and is the key to replace Draize test completely.

The limbal region, located between the cornea and conjunctiva, is the niche for limbal epithelial stem cells (LESCs), which are fundamental for corneal homeostasis and wound healing by supporting cell proliferation and migration. This study aimed to develop and characterize a bovine limbal explant culture model to assess LESC migration and proliferation following exposure to reference chemicals, with a particular focus on corneal reversibility damage.

In this study, limbal explants were isolated from fresh bovine eyes – identical tissue source used in the BCOP assay – and cultured for 13 days. Immunostaining for P63 confirmed the presence of LESC in basal and intermediate cell layers, consistent with previous findings in existing literature. During culture, P63 positive cells formed a monolayer over the stromal surface, proliferating and migrating outward to create a characteristic halo around the explant confirming that LESC migration was occurring.

To quantify this process, the halo-to-explant area ratio was measured as an indicator of LESC migration and proliferation over time. Non-irritating chemicals (No category) did not affect halo formation, while chemicals causing irreversible damage (Category 1) inhibited migration. Importantly, chemicals inducing reversible damage (Category 2) allowed migration and proliferation, although with delayed kinetics.

This novel limbal explant model provides a promising approach to evaluating corneal wound healing and tissue regeneration by tracking LESC migration and proliferation. As a complementary tool, it addresses the limitations of current tests in assessing the reversibility of corneal tissue damage, supporting the transition toward non-animal testing methods in ocular toxicology.

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Wound healing assay to evaluate corneal damage reversibility in SkinEthic HCE model

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Evaluation of chemicals for ocular toxicity is crucial for global product registration and labeling, ultimately safeguarding consumers across all sectors. The traditional Draize rabbit test, used for categorizing irritation severity, is deprecated due to ethical concerns, in addition to low reproducibility and species differences.

Despite big advancements in alternative methods such as OECD TG 492 and 492B, a robust *in vitro* test for assessing damage reversibility remains a critical need. Developing an *in vitro* model for corneal wound healing could address these challenges, benefiting both regulatory compliance and testing ocular therapeutic products. The SkinEthic HCE model presents a promising solution, offering a 3D reconstructed tissue that mimics the structure, morphology, and functionality of the human cornea epithelium. Meanwhile, cell migration emerges as a pivotal factor in corneal wound healing.

Our study aimed to investigate whether the SkinEthic HCE model could effectively assess damage reversibility following exposure to various reference chemicals known to induce different levels of ocular damage. To achieve this objective, a wound healing assay was implemented on the SkinEthic HCE model, incorporating both mechanical damage and chemical exposure to reference substances under the hypothesis that the *in vivo* healing capacity is related to the *in vitro* migration capacity.

Cytotoxicity assays and histological tissue sections of the corneas at 0, 48, and 96 hours provided a detailed documentation of the wound closure process. In summary, the ability to observe damage reversibility in this three-dimensional epithelial model suggests a potential correlation with the four UN-GHS categories. The obtained results highlight the potential of this innovative strategy as the initial step toward a novel human-relevant *in vitro* method for evaluating ocular damage, capable of distinguishing reversal times of the lesion and facilitating classification into all relevant categories without animal use.

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Interleukin-6 as a biomarker of photoirritation: Early steps in establishing a predictive model for photosensitisation

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Daily-use products, such as pharmaceuticals, cosmetic formulations, and fragrances, can contain chemicals that trigger photosensitization reactions when exposed to solar radiation, specifically ultraviolet (UV) light. Although photosensitization is less studied than other adverse reactions, it represents a growing public health concern [1]. Photosensitization includes phototoxicity, also known as photoirritation in topical applications, and photoallergy. These reactions differ in their mechanism: while photoirritation triggers an acute inflammatory reaction, photoallergy involves an immune response. Several methods are available to predict photosensitization; however, no *in vitro* tests currently distinguish between photoirritant and photoallergic substances. Furthermore, the development of non-animal testing methods has become imperative following the implementation of animal testing prohibition in the European Union and many other countries.

For these reasons, we are developing an *in vitro* technique to predict photoirritant or photoallergic substances, focusing on the Key Event 2 – keratinocyte activation – within the Adverse Outcome Pathway for photosensitization [2]. For this purpose, phototoxicity was performed in HaCaT cells following a modified OECD TG 432 protocol [3] testing compounds categorized as photoirritants (PI) and/or photoallergens (PA): Chlorpromazine hydrochloride (PI/PA), Benzophenone (PA) and 8-methoxypsoralen (PI), as well as p-Phenylenediamine as a non-phototoxic allergen.

HaCaT cells were exposed to different concentrations of the test compounds in the absence and presence of UVA light (4 J/cm²), and cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) and Neutral Red Uptake assays. Supernatants collected 24 hours after irradiation were analyzed using a multiplex array with the aim of semi-quantitatively measure and identify inflammatory cytokines levels. Among the cytokines evaluated, interleukin-6 (IL-6) showed notable changes in its release and was subsequently quantified via enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (RayBiotech). A stimulation index (SI) was calculated for both irradiated and for non-irradiated conditions respect to Control, and finally, a Total Stimulation Index was determined as the ratio of irradiated to non-irradiated conditions (SI -Irradiation/SI +Irradiation). The results at the highest tested concentrations showed that 8-methoxypsoralen notably increased IL-6 release (4.08 ± 1.14), in contrast to Chlorpromazine hydrochloride (0.53 ± 0.06), Benzophenone (0.52 ± 0.02) and p-Phenylenediamine (0.67 ± 0.07). These findings suggest that IL-6 could be a biomarker for predicting photoirritation. Fur-



ther studies involving additional compounds classified as photoirritants or photoallergens are needed to validate this biomarker and develop a predictive model.

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A human stem cell-derived test system for agents modifying neuronal N-methyl-D-aspartate-type glutamate receptor Ca²⁺ signaling

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Methods to assess neuronal receptor functions are needed in toxicology as well as for drug development. Human-based test systems that allow studies on glutamate signaling are still scarce for various reasons. To address this issue, we developed and characterized a pluripotent stem cell-based neural culture capable of forming functional networks. Starting from a stably proliferating neuroepithelial stem cell population, we generated “mixed cortical cultures” (MCC) within 24 days. Characterization by immunocytochemistry, gene expression profiling and functional tests (multi-electrode arrays) showed that MCC contain various functional neurotransmitter receptors, and in particular, the N-methyl-D-aspartate subtype of ionotropic glutamate receptors (NMDA-R). As this important receptor is found neither on conventional neural cell lines nor on most stem cell-derived neurons, we focused here on the characterization of rapid glutamate-triggered Ca²⁺ signaling. Changes of the intracellular free calcium ion concentration ([Ca²⁺]_i) were measured by fluorescent imaging as the main endpoint, and a method to evaluate and quantify signals in hundreds of individual cells at the same time was developed. We observed responses to glutamate in the low μM range. MCC responded to kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and a subpopulation of 50% expressed functional NMDA-R. The receptor was modulated by Mg²⁺, Zn²⁺ and Pb²⁺ in the expected ways, and various toxicologically relevant agonists (quinolinic acid, ibotenic acid, domoic acid) triggered [Ca²⁺]_i responses in MCC. Antagonists, such as phencyclidine, ketamine and dextromethorphan, were also readily identified. Thus, the MCC developed here may fill an important gap in the panel of test systems available to characterize the effects of chemicals on neurotransmitter receptors.

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Using the cosmetics animal testing bans as a legislative framework for animal-free scientific innovation

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The European Union bans on animal testing for cosmetics (“the cosmetics bans”), phased in between 2009 and 2013, represent an unprecedented – and arguably as yet unsurpassed – legal milestone in the transition towards animal-free safety testing and research.

A new report by Lush Prize provides an overview of the impact of the cosmetics bans and describes how their legislative framework, with required adaptations, can be extended to ensure animal-free safety and protection for human health and the environment across all regulatory sectors, including industrial chemicals, food, pharmaceuticals and more.

The cosmetics bans are widely recognized as “game-changing” in driving the development of non-animal methods for regulatory safety testing and have “shaped the landscape of alternative methods dramatically”, both in the cosmetics industry and other chemical sectors. They remain a landmark in legal progress to end animal testing, despite challenges presented by other chemicals legislation such as REACH.

Extensive work to develop and approve methods to replace the use of live animals was stimulated to be ready for the deadlines which provided a robust legal framework to end regulatory testing for cosmetic products and ingredients, despite numerous postponements and delays, including legal challenges to stop the bans altogether.

Ultimately however, the final deadline for the ban on remaining animal testing was implemented regardless of available alternatives.

The bans drove a sea change in innovation of non-animal methods, which to various extents replaced animal tests for cosmetics for several key toxicity endpoints, but also exerted positive influence across other regulatory sectors, including industrial chemicals, food, and pharmaceuticals, which also adopted use of these methods and were described as “a unique move that cemented the cosmetics industry’s place as a propeller for innovation in the field of safety testing.”

Over the last decade and following implementation of the cosmetics bans, there has been a significant shift in global recognition to develop, adopt and use non-animal “new approach methodologies” or “NAMs”.

The scientific and commercial opportunities that NAMs offer are infinite, especially where current animal-based test methods are very poor or lacking.

It is widely acknowledged that NAMs are urgently needed as the way forward, with several political and legislative mandates in place over the last few years in both the European Union and the USA to achieve greater flexibility in accepting new safety testing methods and phasing out animal testing to make way for “new and improved” science.

There is a legislative opportunity for a way forward to better science for human health and the environment and there is no reason why governments should not take it.

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Photostability and *in vitro* phototoxicity studies of a promising UVA filter for sunscreens: 1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)-1,3-propanedione

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Moderate sun exposure has been proven to have various health benefits; however, excessive exposure to ultraviolet (UV) radiation is associated with known adverse effects. Between the three UV ranges (UVA, UVB and UVC), UVA is the dominant component (90-95%). Since UVA penetrates the deepest layers of the skin, it induces the formation of reactive oxygen species (ROS), which contribute to cellular damage. Sunscreens play a crucial role in mitigating these harmful effects.

Among the few UVA filters approved in both the United States and Europe, tert-butylmethoxydibenzoylmethane (avobenzene, AVB) is widely used. However, its photochemical instability and incompatibility with certain cosmetic ingredients limit its effectiveness. To address this, the molecule 2-hydroxymethyl-4'-methoxy dibenzoylmethane (DBM11) was synthesized as a potential alternative due to its superior stability under UVA radiation. Initial studies conducted in absolute ethanol under UV irradiation showed negligible decomposition of DBM11, positioning it as a promising candidate for sunscreen formulations.

In this study, DBM11 synthesis was scaled up to enable its incorporation into a model emulsion, and its photostability was evaluated under standardized UV irradiation conditions (ISO 24443) in comparison to AVB. Additionally, an essential aspect of the research was the evaluation of its safety profile through *in vitro* phototoxicity testing, following the OECD Test Guideline 432 (3T3 NRU phototoxicity assay), an established alternative method to animal testing. This assay provides a reliable prediction of phototoxic potential using cultured fibroblast cells, reinforcing the commitment to alternative toxicological approaches in cosmetic safety assessment.

Results demonstrated that DBM11 exhibits superior photostability in emulsified formulations, with slower degradation kinetics compared to AVB, making it a strong candidate for UVA protection. Furthermore, the *in vitro* phototoxicity assay classified both DBM11 and AVB as “probably phototoxic”, indicating a comparable safety profile. DBM11 represents a promising alternative to AVB, offering enhanced photostability while supporting the transition towards animal-free testing strategies in the cosmetic industry.

These findings underscore the importance of integrating *in vitro* methodologies into the evaluation of novel cosmetic ingredients, aligning with the principles of the 3Rs (Replacement, Reduction, Refinement) in toxicology.

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***In silico* and *in vitro* evaluation of the toxicological profile of *Agave durangensis* extract**

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Agave durangensis Gentry is a plant endemic to Mexico, important for the mezcal industry as it is used as raw material in the production of this alcoholic beverage. However, during this process, the leaves are considered agroindustrial waste. For this reason, several studies have been conducted to evaluate their biotechnological potential. In this regard, it has been reported that extracts of this plant exhibit anti-diabetic, antioxidant, antifungal, and antibacterial activities. However, no toxicological studies have been carried out to demonstrate their safety for use. Therefore, we performed *in silico* and *in vitro* studies to determine the ocular toxicity and mutagenic potential of an extract of *Agave durangensis* to understand part of its toxicological profile. For the *in silico* analysis, the SMILE codes of the 10 major compounds identified in the *Agave durangensis* extract by UPLC-MS, which represent more than 90% of the total composition based on their relative abundance, were input into the ADMETLab 3.0 platform. Subsequently, the *in vitro* mutagenic potential was evaluated using the Ames test with *Salmonella typhimurium* strains 98, 100 and 102. Additionally, ocular toxicity was assessed using the Short Time Exposure (STE) test, the Bovine Corneal Opacity and Permeability (BCOP) assay, following OECD 491 and 437 guidelines, as well as an evaluation with the Reconstructed Human Cornea-like Epithelium (RhCE) test using a 3D cornea model reported by da Silva ACG et al., 2020. The *in silico* results for the majority of the components of the extract indicate a medium probability of mutagenicity, a high probability of being an eye irritant, and a medium probability of causing corrosion. However, the *in vitro* evaluation showed that the extract does not induce mutagenesis through any of the mechanisms evaluated across the different *Salmonella* strains. The results of the new approach methodologies focused on ocular toxicity revealed that the extract cannot be categorized according to the Globally Harmonized System, indicating that it does not cause ocular irritation or damage. When comparing the *in silico* and *in vitro* results, a discrepancy was observed. However, a review of the literature reveals that the major component of the extract, which represents more than 60% of the total composition, has not been reported as mutagenic in Ames assays, nor has it been found to cause eye irritation or damage. These findings suggest that *in silico* predictive models are not entirely reliable for predicting these parameters, as the databases used for prediction still contain a limited amount of data compared to the vast number of existing compounds. Additionally, no studies have been focused on these specific toxicological characteristics. Based on these findings, it can be concluded that the *Agave durangensis* extract is neither mutagenic nor irritating to the cornea.

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Study to replace the use of animals in the minimum potency test – Antithrotophic serum

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Introduction: The World Health Organization Guide to the Production and Control of Snake Antivenom recommends the adoption of the 3Rs Principles (Reduction, Refinement and Replacement) by manufacturers of snake antivenom (WHO, 2017). Immunizing horses with antigen preparations (snake venoms) is the first stage in snake antivenom production. After the immunization protocol, the horse's serum is evaluated individually using an *in vivo* test called, in the Vital Brazil Institute Protocol, Minimum Potency test. The function of this test is to assess the neutralizing capacity of the serum and is designed as a Limit Test (Pass x Fail). Once the serum neutralization reaction between the challenge dose of the test venom (5 Lethal Dose) and the specific serum is complete, 06 mice are inoculated intraperitoneally (IP) with 0.5 mL of the solution resulting from the serum neutralization, and the test is completed within 48 hours. The horse is considered approved and released for partial bleeding if the serum has been able to neutralize the lethality of the challenge dose of the specific venom satisfactorily, i.e., if the number of surviving animals among the mice tested is $\geq 50\%$ (≥ 03 live mice). The solutions resulting from the serum neutralization with the samples from the horses in the antithrotophic group show different intensities of turbidity, due to the formation of the Antigen-Antibody Complex, as cited in the 1st Edition of the Brazilian Pharmacopoeia (1926), which adopted the Method described by Vital Brazil as the reference for the analysis of snake antivenom, at that time. This characteristic could be investigated as a possible alternative to the *in vivo* test (Minimum Potency Test).

Objectives: To evaluate the possibility of replacing the Minimum Potency Test with the Optical Density (OD) measurement of the solution after serum neutralization of the serum samples from the horses in the antithrotophic group.

Material and methods: The leftover samples of equine serum and snake venoms from the Minimum Potency Test, which would have been discarded as biological waste, were used as inputs to carry out serum neutralization on a reduced scale, in the same proportions of saline + venom + serum and under the same incubation conditions as the solutions from the *in vivo* test (1 hour at 37°C), considered the Gold Standard. The resulting solution, once homogenized, was transferred in triplicate (150 μ L each) to a 96-well microplate. The OD was read at 405 nm on a microplate reader (Epoch/2 microplate spectrophotometer, Biotek). The averages of the OD triplicates obtained from each horse were calculated and correlated with the results of the corresponding Minimum Potency Test. Thirty-one samples (2024/2025) were analyzed using the free Jamovi software and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), area under the curve (ASC) and the Yuoden Index were calculated at different cut-off points.



Results: The Optical Density (OD) of the 31 samples measured at 405 nm ranged to 0.117-0.831 and the most appropriate cut-off point for pass/fail of the equines was 0.200. This cut-off point gave us 95.65% sensitivity, 75% specificity, 91.67% PPV, 85.71% NPV, 0.891 ASC and 0.7065 Yuoden Index.

Conclusion: The measurement of the OD of the post-serum neutralization solutions of the Antibothropic group samples proved to be a suitable test to replace the Minimum Potency, as it had high sensitivity, specificity, PPV, NPV and ASC when compared to the *in vivo* test (Gold Standard).

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Establishment of readiness criteria for AI-based tools for risk assessment: READYAI

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Recent advances in artificial intelligence (AI), machine learning (ML), and deep learning (DL) are leading to a new era of data-driven innovation, significantly impacting the field of chemical risk assessment. These advanced computational techniques are increas-

ingly being recognized as powerful tools for various applications in toxicology, including data retrieval (e.g., systematic reviews), predictive modeling (e.g., toxicokinetics, quantitative structure-activity relationships (QSARs), read across), toxicity prediction, and evidence integration. By leveraging AI/ML/DL-driven approaches, researchers and regulatory bodies are working toward reducing reliance on traditional animal testing while enhancing the accuracy, efficiency, time- and cost-effectiveness of chemical safety evaluations. To effectively and reliably integrate these tools into regulatory frameworks, it is essential that regulatory authorities, policy-makers, and other stakeholders gain a deeper understanding of their nature, applicability, validity, and inherent limitations. It is equally crucial for computational scientists and AI developers to understand regulatory requirements and prerequisites. This knowledge enables them to develop methods that meet regulatory standards while effectively communicating both the advantages and limitations of these methods for regulatory applications. To maximize the benefits of AI in chemical safety assessment, a skilled and multidisciplinary task force is needed, fostering collaboration among regulators, toxicologists, computational scientists, and AI developers for responsible utilization of AI-driven methodologies. Within this framework, and as part of the Partnership for the Assessment of Risks from Chemicals (PARC) initiative, we have developed the project “Establishment of Readiness Criteria for AI-based Tools for Risk Assessment: READYAI”. This project aims to address the key challenges associated with implementing AI, ML, and DL in toxicology by developing a comprehensive scoring system for evaluating the development, training, and validation of AI-based tools applied in chemical risk assessment. Additionally, it seeks to ensure the consistent application of this scoring system by developers, enabling a more independent and objective assessment of the regulatory applicability of AI/ML/DL-based tools. Key concerns include ensuring the reliability of AI-generated predictions, mitigating biases in training data, improving algorithm interpretability, explainability, reproducibility & transparency, and addressing legal and ethical implications. By defining readiness criteria and adhering to best practice principles, READYAI seeks to facilitate regulatory acceptance of AI/ML/DL tools and promote the responsible application of state-of-the-art AI methodologies on chemical risk assessment.

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Predicting skin irritation, corrosion, and sensitization potential of nanomaterials using OECD-validated new approach methodologies (NAMs)

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Nanomaterials (NMs) are increasingly incorporated into consumer and industrial products, necessitating comprehensive safety evaluations. This study assessed the skin irritation, corrosion, and sensitization potential of five widely used nanoparticles (NPs): titanium dioxide (TiO₂), zinc oxide (ZnO), nickel oxide (NiO), silver (Ag), and polystyrene (PS), using OECD-validated *in vitro* models and Integrated Approaches to Testing and Assessment (IATA). The reliability of OECD Test Guidelines (TGs) 439 and 431 for assessing NM-induced skin irritation and corrosion was confirmed, with results consistent with previous studies. However, regarding *in vitro* skin sensitization assays, including KeratinoSens™ and human Cell Line Activation Test (h-CLAT), produced conflicting results for certain NPs. For instance, TiO₂ and NiO NPs showed borderline or inconclusive outcomes, whereas Ag NPs consistently tested positive across multiple assays. Our findings highlight the limitations of current *in vitro* assays for skin sensitization, particularly for metal-based NPs, underscoring the need for further refinement of New Approach Methodologies (NAMs). Advanced three-dimensional (3D) reconstructed human epidermis (RHE) model demonstrated reduced interference and higher physiological relevance compared to two-dimensional (2D) cell culture systems. Integrating IATA, Defined Approaches (DAs), and NAMs with traditional methods may improve the reliability of NM-induced skin toxicity assessments. This study provides valuable insights into optimizing non-animal testing strategies and enhancing the predictive accuracy of existing *in vitro* models for nanotoxicology.

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Developing a stem cell-based model of neural tube development

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The study of embryonic development is hindered by the difficulty in accessing sufficient embryonic material as well as high costs and, essentially, ethical issues associated with embryo and animal studies. To mitigate these issues, researchers have begun developing embryonic organoids, which represent a New Approach Methodology (NAM) that has revolutionized the study of embryonic development and diseases.

The neural tube (NT) is an essential embryonic structure, as it forms the central nervous system (CNS) of the fetus. During development, the neural plate forms from neuroectodermal tissue and subsequently undergoes folding to form the NT, with any defects in this process resulting in neural tube defects (NTDs), which have significant mortality and morbidity. Rostrally, neuroectoderm destined to become the brain forms from anterior cells that did not ingress at the PS, while caudally, neuroectoderm destined to become the spinal cord forms from neuromesodermal progenitors which form from epiblast cells that did ingress at the PS. However, since the process of neural tube closure occurs 4 weeks post-conception, it is impossible to study this process and resulting NTDs *in vivo*. Animal models are thus routinely used but raise ethical concerns due to the physical harm caused. It is therefore essential to develop *in vitro* models to study this process.

Our approach to study NT development *in vitro* was to form aggregates of mouse embryonic stem cells (mESCs) and pattern them with signaling molecules that stimulate a gastrulation-like process, namely BMP4 and the Wnt agonist Chiron. We did this by either applying the pulse of Chiron/BMP4 to the entire aggregate, or only to part of the aggregate to form a signaling center and therefore a signaling gradient. We then performed ICC for Brachyury to assess PS formation, as well as Brachyury and Sox2 co-staining to assess formation of neuromesodermal progenitors. We additionally performed RT-qPCR to assess the expression of genes involved in NT formation and performed hybridization chain reaction (HCR) to assess the spatial expression of the neuroectodermal gene Sox2.

We found that BMP4 better stimulated Brachyury protein expression than Chiron, although no significant difference was observed transcriptionally. However, the use of signaling molecules was essential to induce Brachyury expression. Additionally, applying Chiron or BMP4 as signaling centers affected the Brachyury expression patterns observed, with signaling centers resulting in polarized Brachyury expression being observed more often, better mimicking *in vivo* embryos. Interestingly, we found that BMP4 pulsing resulted in the highest expression of NT genes, indicating a push towards this lineage, as well as the formation of a neuromesodermal progenitor population, indicating a caudal spinal cord identity being attained.

Overall, these results show the promise of using stem cells to model NT development, which can be furthered into their use to study NTDs and what drugs may contribute towards or mitigate them, without the need for animal models.

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Pain and laboratory animals: Publication practices for better data reproducibility and better animal welfare

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Scientists who perform major survival surgery on laboratory animals face a dual welfare and methodological challenge: how to choose surgical anesthetics and post-operative analgesics that will best control animal suffering, knowing that both pain and the drugs that manage pain can all affect research outcomes. Scientists who publish full descriptions of animal procedures allow critical and systematic reviews of data, demonstrate their adherence to animal welfare norms, and guide other scientists on how to conduct their own studies in the field. We investigated what information on animal pain management a reasonably diligent scientist might find in planning for a successful experiment. To explore how scientists in a range of fields describe their management of this ethical and methodological concern, we scored 400 scientific articles that included major animal survival surgeries as part of their experimental methods, for the completeness of information on anesthesia and analgesia. The 400 articles (250 accepted for publication pre-2011, and 150 in 2014-15, along with 174 articles they reference) included thoracotomies, craniotomies, gonadectomies, organ transplants, peripheral nerve injuries, spinal laminectomies and orthopedic procedures in dogs, primates, swine, mice, rats and other rodents. We scored articles for Publication Completeness (PC), which was any mention of use of anesthetics or analgesics; Analgesia Use (AU) which was any use of post-surgical analgesics, and Analgesia Completeness (a composite score comprising intra-operative analgesia, extended post-surgical analgesia, and use of multimodal analgesia). 338 of 400 articles were PC. 98 of these 338 were AU, with some mention of analgesia, while 240 of 338 mentioned anesthesia only but not post-surgical analgesia. Journals' caliber, as measured by their 2013 Impact Factor, had no effect on PC or AU. We found no effect of whether a journal instructs authors to consult the ARRIVE publishing guidelines published in 2010 on PC or AC for the 150 mouse and rat articles in our 2014-15 dataset. None of the 302 articles that were silent about analgesic use included an explicit statement that analgesics were withheld, or a discussion of how pain management or untreated pain might affect results. We conclude that current scientific literature cannot be trusted to present full detail on use of animal anesthetics and analgesics. We report that publication guidelines focus more on other potential sources of bias in experimental results, under-appreciate the potential for pain and pain drugs to skew data, and thus mostly treat pain management as solely an animal welfare concern, in the jurisdiction of animal care and use committees. At the same time, animal welfare regulations do not include guidance on publishing animal data, even though publication is an integral part of the cycle of research and can affect the welfare of animals in studies building on published work, leaving it to journals and authors to voluntarily decide what details of animal use to publish. We suggest that journals, scientists and animal welfare regulators should revise current guidelines and regulations, on treatment of pain and on transparent reporting of treatment of pain, to improve this dual welfare and data-quality deficiency.

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A dog is a chimp is a mouse is a rabbit? How charismatic species shape lab animal welfare laws and policies

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I'm a veterinarian who has spent his life working with animals in laboratories. As animal welfare laws and policies have evolved over recent decades, I have followed how different species receive differential treatment in law and informally, in practice. In this talk, I explore the positive and negative effects of using charismatic species (such as dogs, primates and rabbits) to advance welfare reforms. Activists present dogs as loving, athletic, and playful, or primates as highly intelligent, and rabbits as sweet and innocent. These perceptions lead to campaigns' successes but also shape the particular policies they enact.

Dogs are the most common animal featured in animal welfare campaigns. From the first English laws in the 1870s, restrictions to help laboratory dogs also benefitted cats and horses and some other animals. In the US, the 1966 Animal Welfare Act focused on pet theft, so applied most forcefully to pet dogs and cats, with minimal protections for other animals. Dogs' image as playful and athletic got them special provisions for exercise in 1985 in the US that applied exclusively to them. Monkeys, rabbits, and others did not get this protection. Primates inspired US laws for psychological well-being, but in law, this and environmental enrichment only applied to primates. Rabbits were the face of campaigns to stop Draize testing, but the funding and refinements that came from this initiative have helped many other species. Mice have experienced the opposite fate as scientists shift their work from other species, for scientific and economic reasons, but also to avoid legal and public scrutiny, counting on people not to care enough about mice to want to protect their welfare.

Do new laws and practices crafted to accommodate interests of one species have a ripple effect that improves the lot of other animals? Or do support special protections for one type of animal at the expense of others? I will show how both outcomes have occurred through the years and argue that activists should be mindful of arguments that elevate species we care about at the expense of those without a human constituency advocating for their interests.

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Quantitative adverse outcome pathway modeling of platinum-induced nephrotoxicity: Developing *in vitro* and *in vivo* models for predictive extrapolation

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Nephrotoxicity is a major concern in the safety assessment of chemicals and drugs, with the kidney being a primary target for toxic effects. Computational modeling, particularly the use of quantitative adverse outcome pathways (qAOPs), offers a promising strategy to improve the translation from *in vitro* to *in vivo*, thereby facilitating more reliable predictions of *in vivo* adverse outcomes and reducing the need for animal testing. Platinum-based drugs are widely used in chemotherapy, yet their clinical application is frequently constrained by nephrotoxic effects. Here, we focus on the development of ordinary differential equation (ODE)-based qAOPs for platinum-induced nephrotoxicity by defining both an *in vitro* and an *in vivo* data-driven model. The *in vitro* model incorporates newly generated, time-course gene expression and propidium iodide (PI) staining data from RPTEC/TERT1 cells exposed to cisplatin. The *in vivo* model employs published rat data, including dose-response platinum kinetics as well as single-dose time-course platinum kinetics, gene expression and histopathology data. Our quantitative approach shows that key processes in the AOP related to immune system activity are non-linear. Specifically, clearance of necrotic kidney cells by immune system activity counters damage accumulation on a timescale of days, yet low-level inflammation still cumulatively affects kidney failure in the long run. Moreover, we perform quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) to link the two models. With this approach, *in vivo* adverse outcome predictions can be made in the future not only for platinum-based compounds but also for the safety assessment of other chemicals and drugs, reducing the need for animal testing.

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A combined *in vitro* and *in silico* strategy to predict toxicokinetics of emerging mycotoxins

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Many mycotoxins that can be produced by fungi and contaminate food and feed are neither regulated nor routinely monitored in food, often due to limited available data concerning their toxicity. These are referred to as emerging mycotoxins and may negatively impact public health. To enhance our understanding of their mode of action and adverse health effects, this study endeavors to predict the toxicokinetics of selected emerging mycotoxins produced by *Alternaria* and *Fusarium* spp., aiming to predict internal exposure concentrations at organs of toxicological concern. To pursue this goal, Physiologically Based Kinetic (PBK) models as an alternative method to animal experimentation were developed based on a recently established model concept. While physiological parameters could be extracted from literature, quantitative structure-activity relationship (QSAR) tools were employed to predict tissue partitioning and gastrointestinal uptake. Kinetics of phase I and II hepatic metabolism were assessed *in vitro* by following emerging mycotoxin biotransformation in incubations with rodent and human liver S9 and microsomal fractions, using LC-MS/MS analysis. Therewith, rodent PBK models for selected emerging mycotoxins were parameterized and their predictive accuracy was evaluated against already available toxicokinetic data from animal studies. Then, we performed cross-species extrapolation to develop human PBK models and used those to estimate mycotoxin concentrations in organs of toxicological relevance. These developed quantitative and robust tools will be used for *in vitro* to *in vivo* extrapolation to support next generation risk assessment of emerging mycotoxins.

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Development of an *ex vivo* canine model for deep pyoderma: Synergistic effect of bacteriophage and fusidic acid against methicillin-resistant *Staphylococcus pseudintermedius*

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In the 21st century, responsible use of antibiotics is essential to counter the growing threat posed by multidrug-resistant pathogens. Considering this challenge, new approaches for the treatment of bacterial infections are crucial. Recently, the combination of antibiotics and bacteriophages (phages) has opened a promising avenue for combating multidrug-resistant bacteria. The synergistic effect of these two therapeutic modalities could provide an effective strategy for treating infections that are difficult to control with conventional treatment.

The first objective of the study was to develop a canine *ex vivo* model that simulates deep pyoderma, specifically caused by methicillin-resistant *Staphylococcus pseudintermedius* (MRSP). This model aimed to replicate the clinical conditions of deep skin infections in dogs, which are often difficult to treat. The second objective was to utilize this *ex vivo* model to assess the topical antimicrobial efficacy of a newly isolated phage. Additionally, the study aimed to investigate the potential synergistic effect between this bacteriophage and fusidic acid, a topical antibiotic, in combating MRSP infections.

For the model, skin of the lateral thorax from six dogs of telogen breeds was harvested after euthanasia within 24 h. The skin was clipped, the subcutis removed and epidermis cleaved via modified salt split technique. The *ex vivo* deep pyoderma model was established in Franz diffusion cells where the skin was inoculated with 1x10⁷ colony-forming units of a clinical MRSP strain for 16 h. Experimental groups were treated with fusidic acid 0.4 mg and phage vB_SpsS_LmqSK144-4 at a concentration of 2x10⁶ plaque-forming units alone or in combination for additional eight hours. Histopathology showed the bacteria reaching the superficial and perifollicular dermis. Co-treatment of fusidic acid and phage significantly reduced the concentration of MRSP after eight hours.

In conclusion, this model has the potential to serve as a valuable resource for evaluating topical alternatives to systemic antibiotics, as well as exploring their potential interactions in the treatment of canine deep pyoderma. The use of phages in combination with topical treatments could offer a promising approach for managing skin infections caused by MRSP in dogs.

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Prevalidation results from GARDskin for medical devices: *In vitro* sensitization testing of medical device extracts for implementation in ISO 10993-10

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Biocompatibility testing is an important part of the safety assessment of medical devices (MD) to ensure compatibility with biological systems and to predict potential adverse reactions. Toxicological endpoints that need to be evaluated for nearly all MD include cytotoxicity, irritation and skin sensitization. Testing for the two former endpoints is partly conducted *in vitro*. While the regulatory acceptance of New Approach Methods (NAMs) for skin sensitization testing of neat chemicals has significantly reduced the need for animal testing in a variety of industrial sectors, there has been a slower uptake of NAMs in the MD sector for this endpoint, and it is estimated that more than 30,000 animals are used every year only to meet the information requirements in EU alone.

The slower uptake of NAMs in the MD sector can partly be attributable to technical challenges associated with testing of extracts from solid or semi-solid materials, as well as a lack of specific validation of these methods for MD testing. However, efforts at ISO level have led to the publication of the Technical Specification (TS) document 11796, which provides a framework to guide assay developers in evaluating the applicability of candidate methods for MD testing.

The work presented herein describes the work to adapt and validate the protocols of the GARDskin assay (OECD TG 442E) for testing MD according to the guidelines in the TS. To enable MD testing and comply with established standards, the GARDskin protocol has been modified to include a pre-sample treatment procedure involving extractions in polar (saline) and non-polar (olive or sesame oil) vehicles according to ISO 10993-12.

Here, results from two separate studies are presented. In the first study, a silicone material (MED-2000) and a TPU material were spiked with a variety of sensitizers (n = 4) during material production. Extracts were prepared in polar and non-polar solvents and tested as described above. In the second study, a negative control material (MED-2000) was extracted in both polar and non-polar vehicles and spiked with a panel of sensitizers and non-sensitizers (n = 16, 3 replicates) as described in the TS.

Based on the results of the first study, all spiked materials were correctly identified as skin sensitizers in both extracts, while the medical-grade control materials were accurately classified as non-sensitizers, demonstrating that the assay is compatible with both polar and non-polar vehicles and sufficiently sensitive to detect sensitizers in complex extracts from MD. In the second study, preliminary results showed a strong correlation between the OECD protocol for testing neat chemicals and the MD protocol for testing spiked extracts, with 88% (14/16) concordant classifications. Additionally, the assay achieved an accuracy of 94% (15/16) and 100% sensitivity, further confirming that false negative results are not a concern. Based on the outcome of the studies reported in this work, the GARDskin Medical Device (MD) protocol has qualified for the final step in the TS and is ready to enter the ring trial study to conclude on assay transferability.



In conclusion, GARDskin MD is currently the only *in vitro* assay fulfilling the requirements in the TS of being both OECD validated and compatible with testing in polar and non-polar solvents. This would ultimately pave the way for its adoption into ISO 10993-10, offering a much-needed alternative to traditional animal testing.

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In vitro safety evaluation of ozonized oils

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Ozone is an unstable inorganic gas characterized by its high reactivity and oxidizing power. Upon contact with organic fluids, it generates reactive oxygen species (ROS), which can react with cellular components of microorganisms, causing damage that compromises vital functions and leads to cell death. Ozonized vegetable oils are obtained through ozonation processes. In these oils, ozone reacts with double bonds of fatty acids, forming ozonides and peroxides such as hydroperoxides, hydrogen peroxide, and other organic peroxides and aldehydes. These products facilitate handling and stabilize O₃, being widely used in medical and cosmetic therapies for topical application. Their activity can be further improved through nanoemulsions that aid in the gradual and controlled release of ozonides, ensuring adequate stability, modulating correct permeation and improving sensory characteristics. Few studies have evaluated the toxic potential of ozonated oils and their nanoformulations. These evaluations are essential for making new therapies available on the market and ensuring consumer safety. The studied oils and nanoemulsions have already demonstrated superior antimicrobial potential compared to conventional treatments. Therefore, it is extremely important to investigate the risks related to these therapies. Therefore, the objective of this study was to evaluate *in vitro* the cytotoxicity of ozonized vegetable oils derived from sunflower and olive oils, on mouse embryonic fibroblast cells (3T3) and immortalized human keratinocytes (HaCaT). Initially, to assess the effect on cytotoxicity, the cells were incubated with increasing concentrations of ozonized oils (50 to 5000 µL/mL) for 24 hours. Subsequently, cell viability was evaluated using the MTT reduction and neutral red uptake assays. Both oils did not show a reduction in cell viability of 3T3 cells at low concentrations. However, the oils significantly reduced cell viability at the highest tested concentration (5000 µL/mL) as measured by the MTT method. These results demon-

strate the safety of ozonized oils but underscore the importance of further studies to elucidate the safety limits and mechanisms involved in the observed cytotoxic effects at high concentrations. The perspectives of this study include further evaluation of the potential cytotoxic and genotoxic effects of nanoemulsions derived from ozonized oils. Future investigations aim to elucidate the underlying mechanisms and safety thresholds associated with these effects, contributing to a comprehensive understanding of their biological impact and therapeutic applicability.

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Not yet nixing rabbit eyes from the six: The TTT (OECD test guideline 492B) is not predicting the full range of in vivo eye irritation classifications for agrochemical formulations

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Since its adoption in 2015, the EpiOcular™ eye irritation test (EIT, OECD test guideline (TG) 492) has been the *in vitro* method of choice to identify agrochemical formulations non-irritant to the eye. For serious eye damage, however, none of the *in vitro* test methods including the bovine cornea opacity and permeability test (BCOP, OECD TG 437, and protocol modifications), the isolated chicken eye test (ICE, OECD TG 438), the ocular irritation assay (OECD TG 496) or the hen's egg test on the chorioallantoic membrane (HET-CAM) assay proved sufficiently sensitive to correctly identify agrochemical formulations classified as seriously eye damaging *in vivo*. In 2022, the OECD adopted a new *in vitro* test guideline for identifying chemicals inducing serious eye damage, eye irritation, and chemicals not requiring classification for eye irritation (OECD TG 492B). However, within the formal validation study of this time-to-toxicity (TTT) test method, agrochemical formulations were not included. To close this gap, 25 agrochemical formulations with historical *in vivo* data were assessed in the TTT. The overall accuracy of the TTT was 52% when using the three-class-prediction model with 0%, 90% and 50% of the *in vivo* Cat 1, Cat 2 and not classified formulations correctly predicted, respectively. Therefore, to facilitate non-animal-based identification of seriously-eye-damaging agrochemical formulations, testing protocols and/or prediction models must be revised or classification guidelines should be adjusted to align with human-relevant *in vitro* testing rather than relying on *in vivo* Draize data as a reference point.

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Technical challenges of testing polymer materials by OECD test guideline methods addressing effects on human health, on biotic systems and analytics

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As part of the European Green Deal and Chemical Strategy for Sustainability, REACH registration of polymers is expected and may require analytical, ecotoxicological and toxicological data. Regulatory testing is per default using OECD test guidelines (TG) methods and preferentially non-animal or new approach methods (NAMs). Typically, these TGs were developed and validated based on small molecules and their technical applicability as well as predictive capacity is not comprehensively evaluated for polymers. In contrast to small molecules, however, polymers are characterized by high molecular weight and distribution of chain lengths. This makes them mixtures of different molecules; many of which are not readily soluble and hence present a challenge to test systems in aqueous media. Therefore, the technical applicability of the existing TGs on polymers is potentially limited and involves the risk of generating unreliable data impacting risk assessments.

Using a set of 17 polymer materials we assessed the technical applicability of several OECD TG methods. The determination of the solubility of the polymer materials was measured according to OECD TG 120. The polymer materials were tested in water, cell-culture media and on air-liquid interface (for OECD TG 202, daphnia sp. acute immobilization test, OECD TG 487, *in vitro* mammalian cell micronucleus test and OECD TG 439, *in vitro* skin irritation: reconstructed human epidermis (RhE) test, respectively). The sample preparation procedures outlined in these TG were not universally applicable. For water-soluble and dispersible polymers, the OECD TG 202 protocol can generally be applied. For reactive and poorly soluble polymers uncertainties remain, e.g., if the organisms have been exposed to the actual polymer, and if concentration control analyses can be reasonably applied. For the *in vitro* micronucleus assay, the protocol described in OECD TG 487 can only be applied for water soluble polymers. The application of undiluted polymer materials to the surface of RhE as described in OECD TG 439 is technically feasible, but polymer materials in the physical form of waxes and foams could not be tested without special sample preparation.

We present here challenges to testing polymer materials by OECD test guideline and propose amended approaches which are more suitable for polymers. This is merely cautioning the indiscriminating use of OECD TG for polymer materials and is highlighting the need to develop adapted and standardized methods for analytical, ecotoxicological and toxicological evaluations of polymers.

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Dataset development and labeling for algorithm training applied to facial recognition of non-human primates *Sapajus apella* housed in a research animal facility

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The scientific community must ethically adhere to the 3Rs Principle to ensure that, alongside biomedical studies refined by procedures and techniques that maximize animal welfare, methods are also developed to enable the reduction and replacement of animals. In this context, the application of artificial intelligence (AI) offers promising opportunities for developing and implementing innovative technological solutions that assist both in animal management and in the accurate and objective assessment of animal welfare.

Artificial intelligence has also been employed to develop facial recognition software aimed at identifying individuals. This technology has already been applied to some non-human primate species, including guenons, chimpanzees, gorillas, and rhesus macaques, but with the purpose of individual identification in the wild rather than pain recognition, achieving high success rates. However, the facial recognition software developed so far is not commercially distributed for use in research animal facilities that house non-human primates.

The project "Development of AI-Based Technological Solutions for the Welfare Assessment of Animals Used in Biomedical Research" aims to develop facial recognition software for the individual identification of non-human primates used in biomedical research. This software seeks to eliminate the need for tattoos and provide a non-invasive identification technique, enhancing colony management refinement.

A total of 4,458 high-resolution photographic records (3768 x 2736) were collected in both lateral and frontal side faces from five adult male and female non-human primates from a colony of 20 *Sapajus apella* individuals maintained at the Carlos Chagas Filho Biophysics Institute of the Federal University of Rio de Janeiro (UFRJ). The goal was to develop a software prototype.

For dataset cleaning and organization, all images were individually inspected, and low-quality images were discarded. The dataset was structured by correlating photos with a spreadsheet containing annotations on the photographed animals' characteristics (identification number, sex, age, etc.), hosted in the Amazon SageMaker Ground Truth cloud environment to create a machine learning training dataset. Image labeling and annotation were performed using the Labelbox platform to enable facial recognition of *Sapajus apella* individuals.

Once the dataset was created, the labeled data (in JSON format) were pre-processed for machine learning training. So far, no dataset or facial recognition software for this species has been reported, either in the wild or in research facilities. This software development represents a ground-breaking initiative for identifying captive capuchin monkeys (*Sapajus apella*) for scientific research or zoological purposes, positioning itself as an alternative and non-invasive identification method, surpassing current conventional methods.

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Comparison of QSAR models for genotoxicity and carcinogenicity prediction of cyanobacterial secondary metabolites

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Cyanobacterial secondary metabolites are considered to have great potential as biologically active compounds and are attracting great interest in the development of several types of agents, including biopesticides. They are an interesting alternative to traditional pesticides because of their specific mode of action, less probability of resistance and their sustainable characteristics. However, even with desirable characteristics, these compounds have to undergo the regulatory pathway of toxicological evaluation for registration and commercialization. Due to the lack of information on their toxicological properties, the use of *in silico* methods could be efficient tools to screen the secondary metabolites for their future use. In the present study, 30 cyanobacterial secondary metabolites were chosen from a database based on MW, and their genotoxic and carcinogenic properties were screened using Quantitative Structure Activity Relationships (QSARs) methodologies, which are mathematical models that predict properties of a chemical based on its structural characteristics. The *in silico* platforms employed were VEGA, OECD QSAR Toolbox, and TEST (Toxicity Estimation Software Tool). In order to score the predictions of each model of the above platforms, the majority consensus approach was applied which assigns scores 0 and 1 for negative (no alerts) and positive (presence of alerts), respectively. The average results were classified as mutagenic potential (> 0.5), or non-mutagenic potential (< 0.5). Scores of 0.5 were considered inconclusive. Genotoxicity was evaluated using 4 models for point mutation (Ames test) and 5 models for structural mutations (chromosome aberration and micronucleus test). Carcinogenicity was predicted by four models included in the VEGA platform. Analysis of genotoxicity point mutation results showed genotoxicity alerts for 4 compounds and no alerts for 18 compounds. For chromosomal aberration, genotoxicity alerts were found for 9 compounds, while alerts were not predicted for 15 compounds. The carcinogenicity models predicted just 17% of compounds to have no alerts, while 15 compounds (50%) were predicted to have at least one alert, with the limitation that only 8 out of 15 (53%) were into the AD (have strongly similar compounds with known experimental values in the prediction). Eight compounds (27%) led to inconclusive results for point mutation and 6 (20%) for structural mutations. Inconclusive carcinogenicity prediction was 30% of the searched substances. Although more literature information regarding the selected compounds would be desirable, there are gaps in knowledge about these metabolites and the understanding of their mechanisms of action remains limited. In conclusion, while biopesticides based on cyanobacteria hold great potential for sustainability, addressing the current limitations through ongoing research and development efforts is essential for their successful integration into different fields.

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Environmental cues in 3D models: A path to more physiologically relevant cultures

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Replacing animal testing requires physiologically relevant *in vitro* models that faithfully replicate the complexity of human tissues. Traditional 2D cultures lack essential microenvironmental cues such as cell-cell interactions, diffusion gradients, and biomechanical cues, making them poorly suited for studying advanced disease mechanisms and therapeutic responses. 3D culture offers a more representative alternative, but its success depends on precise environmental control and standardized methodologies to ensure reproducibility and translational relevance.

Despite its importance, environmental regulation is often overlooked in *in vitro* culture, leading to discrepancies with *in vivo* conditions. Shear stress, commonly encountered in suspension-based 3D culture systems, disrupts cellular organization, alters gene expression, and affects differentiation pathways. Oxygen availability is another critical factor, influencing metabolic adaptation, stemness, and drug response. Tumor microenvironments are characterized by hypoxic gradients that drive cancer cell behavior, yet standard incubators fail to replicate these conditions, exposing cells to artificially high oxygen levels that do not reflect physiological reality.

Using the MDA-MB-231 breast cancer cell line, we examined how oxygen regulation and optimized 3D culture conditions shape tumor-like behavior. Morphological analysis revealed distinct structural differences, with hyperoxia inducing multinucleated clusters absent under physiological oxygen levels. ATP assays confirmed metabolic shifts, with ATP concentrations significantly higher in hyperoxia (75,197 μ M) compared to normoxic conditions (30,507 μ M). Spheroids transitioning from physiological oxygen levels to hyperoxia exhibited rapid metabolic adaptation, underscoring the dynamic impact of oxygen availability on tumor metabolism.

Beyond oxygen regulation, shear stress plays a crucial role in cancer modelling, affecting cellular architecture, proliferation, and metastatic potential. While physiological shear stress in breast tissue ranges from 0.01 to 3 Pa, many 3D culture systems expose cells to excessive forces, altering natural behavior. Our spheroids were cultured under conditions maintaining shear stress within the physiological range with a maximum of 0.014 Pa, ensuring a more accurate representation of *in vivo* tumor mechanics.

Improving environmental control in 3D models is essential to advancing research and developing more reliable *in vitro* systems. A deeper understanding of oxygen regulation and biomechanical factors can enhance the physiological relevance of *in vitro* models, improving their translational value for cancer research and therapeutic screening while reducing reliance on animal models.

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Tackling sex bias in next generation risk assessment with women-specific physiologically based kinetic modelling

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Ensuring the applicability of next-generation risk assessment (NGRA) to the physiology of the entire human population is essential for enhancing its relevance and fostering its adoption over traditional animal-based approaches. For elucidating the absorption, distribution, metabolism, and excretion (ADME) processes of xenobiotics, physiologically based kinetic (PBK) modeling offers an enabling framework that combines physico-chemical with human biological properties and integrates diverse data sources to simulate chemical disposition across biological compartments. However, current PBK modeling methodology largely reflects historical research biases, predominantly emphasizing standard male physiology while neglecting essential sex-derived differences. As a result, risk assessment neglects tissue that is relevant for women and often fails to capture critical health outcomes, particularly in women. Here, we present a PBK modeling strategy that explicitly represents women's physiology. We incorporated female-specific parameters for aspects such as body composition, enzyme expression levels and dynamic processes. Furthermore, we evaluated predicted outcomes with regards to known cases of drugs causing adverse effects attributed to sex-dependent pharmacodynamic differences. Additionally, this framework extends the predictive scope of new approach methodologies (NAMs) by modeling compartments of major interest for women's health (breasts, uterus, ovaries), based on detailed anatomical and histological composition data. This work is a crucial step toward developing *in silico* modeling methods for female-specific conditions and better understanding their role in modulating adverse health outcomes induced by xenobiotics, particularly in relation to the menstrual cycle, menopause, pregnancy, and certain gynecological diseases.

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Leveraging innovative research tools to meet public health challenges: Key insights from the EU BioMed21 workshop

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The landscape of biomedical research is undergoing a fundamental shift towards human-centric, interdisciplinary approaches designed to enhance translational relevance, maximize research impact, and address urgent public health challenges and unmet medical needs (UMNs). Across Europe and internationally, there is growing momentum to develop and implement non-animal methods (NAMs) that improve research translatability while complementing or replacing animal-based models. Achieving this transition requires effective cross-sector collaboration to align priorities, harmonize strategies, and ensure that policy decisions are driven by robust scientific evidence, ultimately benefiting both public health and society.

Against this backdrop, on November 18, 2024, Humane Society International/Europe (rebranded as Humane World for Animals as of February 14, 2025), on behalf of the Biomedical Research for the 21st Century (BioMed21) initiative, organized a high-level science policy workshop, "Leveraging Innovative Research Tools to Meet Public Health Challenges." This workshop brought together diverse interest-holders to identify priority areas where multidisciplinary efforts are most needed. Discussions focused on harnessing innovative technologies to address UMNs, rethinking research infrastructure to ensure long-term resilience, and advancing human-centric tools to drive meaningful policy transformation. Key themes included the societal impact of research funding, the need for standardization, and the importance of systems-based solutions to address interconnected challenges.

This presentation synthesizes the key insights and outcomes from the workshop, outlining a roadmap for advancing science-driven, health-focused biomedical research. Specifically, fostering progress in biomedical research and healthcare requires:

- Early and sustained engagement with patients and stakeholders to ensure alignment with real-world needs
- Cross-sector collaboration spanning academia, industry, regulatory bodies, and international partners
- Regulatory acceptance and global standardization to build trust in novel technologies
- Sustainable, long-term funding models that support technology qualification, validation, and infrastructure development
- High-quality data sharing guided by FAIR principles to enhance transparency and reproducibility
- Comprehensive education and training to cultivate a cross-disciplinary workforce
- A commitment to sustainable and equitable healthcare, addressing disparities and ensuring accessibility for underserved populations

By embracing these principles, the biomedical research community can drive transformative change, ensuring that emerging innovations translate into tangible health and societal benefits. These guiding principles can be used for shaping future funding initiatives, such as the upcoming EC FP10.

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3D cell culture aligned with the 3Rs principle: Development of a stamp-based agarose microwells system for advanced cell culture

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Introduction: Three-dimensional (3D) cell culture provides a more accurate representation of the *in vivo* microenvironment compared to the conventional two-dimensional (2D) cultures by enhancing cell-cell and cell-matrix interactions under physiologically relevant conditions. This advancement is crucial for overcoming the limitations of traditional cultures, enabling the development of more representative experimental models for both Basic and Applied Research. Spheroids and organoids, 3D cellular structures composed of specific cell types, are widely used in Biomedical Research due to their ability to replicate the spatial organization and key functional aspects of organs. These models facilitate disease studies, drug testing and tissue engineering applications. **Objectives:** This study aimed to develop an efficient, cost-effective, and reproducible methodology for generating spheroids and organoids using a stamping system to create microwells in agarose, aligning with the 3Rs principles (Reduction, Replacement, and Refinement) in animal research. **Materials and Methods:** A novel stamp was designed to create 663 microwells per well of six-well trays, providing a standardized and controlled environment for cell aggregation. Primary porcine pancreatic islet cells were seeded into these microwells and incubated at 37°C with 5% CO₂, with culture medium changes every three days. The formation of 3D structures was periodically monitored, and samples were collected for morphological and functional characterization. **Results:** The proposed methodology enabled the generation of uniform, high-quality 3D structures with stable architecture. Reduced experimental variability and minimized cell manipulation were observed, promoting efficient cell interactions and the formation of organized microenvironments. The agarose microwells produced using the developed stamp proved to be a simplified, cost-effective and efficient solution for 3D cultures, ensuring experimental reproducibility. **Discussion:** This research aligns with the mission of advancing alternatives to animal use in Research and Education in the Life Sciences. The study was recently accepted for publication in an International Scientific Journal with both video and text formats, highlighting its contribution to improving scientific Education and Research dissemination. Additionally, the establishment of the Biotechnology and Innovation Center (NBI) at DTAPEPI, integrated into the PREMIUM Network Program at the University of São Paulo

Medical School (FMUSP), reflects an institutional commitment to the 3Rs principles by providing state-of-the-art facilities and technologies for both internal and external researchers. **Conclusion:** This approach offers a standardized and cost-effective promising solution for developing 3D culture models, with applications in Basic research, drug testing, and Tissue engineering. The isolation and culture of porcine islets were approved by the Animal Ethics Committee (CEUA-FMUSP) under protocol number 1699/2021.

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Forwarding & validating *in vivo* translational digital biomarkers for translation, reduction, & refinement

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Emerging technologies that provide continuous monitoring of animals in their home cages offer immense opportunities to improve research animal welfare, scientific data quality, and translation. They offer key advantages over traditional methods of measuring animal behavior and physiology that are limited by infrequent sampling and stressful environments, potentially compromising wellbeing and research outcomes. However, the adoption of these technologies is not yet widespread due to a number of barriers.

Therefore, to assist in advancing these impactful technologies, the 3Rs Collaborative's Translational Digital Biomarkers (TDB) initiative has united stakeholders through shared experiences and thought leadership. Furthermore, to ensure the reliability and relevance of digital biomarkers, they have adopted a variation of the Digital Medicine Society's (DiMe) V3 Validation Framework for preclinical contexts. This approach emphasizes verification, analytical validation, and clinical validation to ensure the reliability, precision, and applicability of digital biomarkers. We will present key considerations for engaging in digital biomarkers and the approach for their verification.

Ultimately, by fostering interdisciplinary collaboration and integrating robust validation processes, we can accelerate the adoption of new techniques, ultimately advancing the 3Rs and translational research and ensuring research animals benefit from technological evolution.

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Imaging-based *in vitro* methods: Challenges and opportunities for regulatory acceptance

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Imaging technologies are increasingly employed in biomedical research and experimental toxicology to extract detailed morphological and functional information from cellular models. These technologies offer unique insights into cellular dynamics, differentiation pathways, and responses to external stimuli, presenting a significant opportunity to integrate imaging-based *in vitro* methods into international regulatory guidelines. This integration would address the growing demand for human-relevant data in safety and efficacy assessments.

However, translating imaging technologies from research and development (R&D) to international regulatory acceptance requires coordinated efforts. The scientific community must establish standardized protocols under robust quality management systems, ensure inter-laboratory transferability, and demonstrate the reliability and reproducibility of imaging-derived data. In the context of *in vitro* methods, challenges include capturing high-resolution spatiotemporal changes during cellular differentiation, identifying relevant imaging biomarkers, and managing the large, complex datasets generated by high-content imaging modalities.

Here, we discuss the current challenges associated with imaging-based *in vitro* methods, including image acquisition, data analysis using advanced computational tools such as artificial intelligence, and data management. We emphasize the need for a tailored strategy to enhance data quality, reproducibility, and comparability. Additionally, we outline key considerations for designing and implementing future transferability and validation studies to facilitate the regulatory acceptance of imaging-based *in vitro* methods.

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Advancing alternatives to animal use in Brazil: Regulatory progress, statistics, and public opinion

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More than 10 million animals were used for research purposes in 5 years in Brazil, a country where it is a crime to use animals when alternatives exist, the use of recognized alternative methods is mandatory,

and most of the population agree that the country should reduce the use of animals. Regarded as the leading country Latin America, Brazil has made substantial progress on establishing ethical standards in animal research and advancing alternatives to animal use in research and testing in the last decades. Brazil's Constitution states that the government must protect animals from cruelty. According to the Environmental Crimes Law, it is a crime to subject animals to harmful or cruel experiments, even for education or scientific purposes, when alternatives exist. Law No. 11,794/2008 regulates the use of animals in education and scientific research, creates the National Council for the Control of Animal Experimentation (CONCEA), and mandates the establishment of Ethics Committees for the Use of Animals (CEUA), including a representative of animal protection society in all institutions that use animals. CONCEA has already recognized more than 40 alternative methods, making their use mandatory 5 years after the recognition; however, a monitoring system has not been implemented. Regarding cosmetics animal testing, there are several state bans and CONCEA introduced a partial national ban in 2023. While 79% of the population agrees that Brazil should have a national ban on animal testing and sales of animal tested cosmetics, a federal bill has been under discussion in Congress for over a decade and still awaits a vote. The efforts of the Humane World for Animals (formerly known as Humane Society International) to reduce animal use in the safety assessment of school supplies resulted in updated national regulations (ABNT NBR 15236/2021; Inmetro Ordinance 423/2021) recommending an ingredient-based approach and ensure the use of animals only as a last resort. Despite several requests for a similar toy's regulation update (and the fact that 85% of the Brazilian population agrees that safety of these products should be assessed by non-animal methods), the regulation in force is still based on finished product testing and has no language on the use of animals only as a last resort for acute oral toxicity. As the current CONCEA data collection system does not require reporting the purpose of animal use, it is not possible to identify how many of the almost 2 million rodents used from 2019 to 2023 were subjected to safety tests that could be avoided. The chemicals scenario is promising, as the recently sanctioned Brazil's chemical law limits the use of animals only as a last resort and provides for the creation of a strategic plan to promote alternatives. Regarding biomedical research, 90% of the Brazilian population agree that the country should reduce the use of animals in research, according to a public survey conducted in 2024. Some initiatives to promote the recognition and implementation of non-animal methods have emerged, but Brazil has not yet committed to phase out animal use nor established a roadmap to accelerate the reduction of animal use in biomedical research. Brazil faces unique challenges and opportunities in advancing alternatives to animal methods, and a multi-stakeholder joint effort is needed to achieve international alignment while responding to the increasing demand of the Brazilian society for an animal cruelty free culture in research and testing.

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A new use of Optisol-GS in bovine cornea preservation for the BCOP assay

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Introduction: Ensuring the safety of products that cause ocular irritation is essential. The Bovine Corneal Opacity and Permeability (BCOP) test is an accepted alternative method for ocular irritation assessment in regulatory contexts. Although it is an ethical alternative to the Draize test, its implementation faces challenges such as difficulties in cornea acquisition and storage, high costs, and the need for standardization. Improvements in cornea preservation and histopathological evaluation are necessary to expand its application and sustainability.

Objective: This study aims to evaluate the efficacy of cold preservation of bovine corneas for the execution of the BCOP test with proficiency chemicals, seeking improvements that could extend corneal use and ensure the method's sustainability by utilizing enhanced histopathology, strengthening the commitment to promoting more ethical and sustainable alternatives to animal use in scientific research.

Method: Experimental tests were conducted on bovine corneas treated with Optisol-GS, HBSS, and EMEM at different experimental time points and challenged with nine proficiency chemicals according to the OECD Test Guideline 437 – BCOP. Histopathological analysis was performed following the methodology described by Jeong et al., 2022.

Results: Optisol-GS preserved bovine corneas for up to 7 days without affecting BCOP results, maintaining IVIS classification consistency across proficiency chemicals. Histological analysis confirmed tissue integrity, with minimal epithelial and stromal alterations compared to fresh samples. Based on the gradual histological analysis, ocular irritation increased according to the degree of irritation of the tested chemicals. Compared to HBSS and MEM, Optisol-GS demonstrated superior preservation, reducing corneal opacity and endothelial damage, supporting its suitability for extended storage in BCOP applications.

Conclusions: The commercial solution Optisol-GS has demonstrated a novel application for the preservation of bovine corneas in the BCOP assay for up to seven days. This finding grants the study scientific merit for its innovative contribution to the field, advancing knowledge on corneal preservation for the BCOP assay and benefiting both research practice and the scientific community.

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From waste to wonder: Combining alternative methods and sustainable practices for safe and effective cosmetic ingredients

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The cosmetic industry faces the dual challenge of ensuring ingredient safety and efficacy while adopting sustainable and ethical practices. The prohibition of animal testing worldwide has accelerated the adoption of New Approach Methodologies (NAMs) and Next Generation Risk Assessment (NGRA) strategies to assess toxicological profiles. Simultaneously, sustainable initiatives such as upcycling enable the valorization of by-products, transforming waste into high-value cosmetic actives. This study presents an integrated approach that combines advanced *in vitro* safety assessment with cutting-edge efficacy evaluation techniques for an upcycled antioxidant-rich ingredient designed for skincare applications identified as LLT23. For safety assessment, a battery of *in vitro* tests was conducted to address key toxicological endpoints. Following OECD Guidelines 429, 491, and 492, the ingredient was classified as “non-irritant” for dermal and ocular applications. The phototoxicity assessment (OECD 432) confirmed no phototoxic potential. Genotoxicity screening via Ames test (OECD 471) yielded negative results, while the micronucleus test (OECD 487) was inconclusive. To resolve this, the ToxTracker assay validated the non-genotoxic classification. Skin sensitization tests provided conflicting results (OECD 442C = positive, OECD 442E = negative), leading to the application of GARDskin Dose-response testing, which predicted the corresponding human NOEL (No Observed Effect Level) value 948,000 µg/cm² and allowed the definition of a safe dose using Quantitative Risk Assessment 2 approach. For efficacy evaluation, a 3D skin model coupled with RNA-Seq technology was employed to investigate the ingredient's impact on gene expression. Two concentrations (0.05% and 0.1%) were tested. The lower concentration had minimal effects, modulating only 16 genes while at 0.1%, the ingredient significantly altered the expression of 804 genes, indicating diverse skin benefits. Functional analysis demonstrated its potential to enhance cell regeneration and renewal, promote detoxification, provide antioxidant defense against oxidative stress, strengthen skin barrier integrity, and stimulate collagen synthesis. This study highlights the power of integrating NAMs for safety assessment with RNA-Seq-driven efficacy testing to develop high-performance, sustainable cosmetic ingredients. The results reinforce the viability of upcycling as a strategy to create effective and environmentally responsible skincare solutions. By leveraging alternative methodologies and sustainable innovation, this approach contributes to the future of ethical and scientifically robust cosmetic product development.

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Advancing *in vitro* fish chronic toxicity testing for cosmetic ingredients: Early adaptation of the RTgill-W1 cell line assay for chronic tests

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The cosmetic industry seeks to enhance the environmental safety of cosmetics, requiring hazard data. However, ecotoxicity data, especially on chronic toxicity, is largely lacking. Due to animal welfare regulations, ecotoxicity methods must be animal-free. This study aimed to adapt the RTgill-W1 cell line assay (OECD TG 249) for long-term culture to further be used as an *in vitro* alternative for chronic fish toxicity testing. The feasibility of maintaining RTgill-W1 cells for 36 days was evaluated using different culture media (L-15/ex, L-15 + 1% fetal bovine serum (FBS), and L-15 + 4.7% FBS). Cells were seeded in 24-well plates (2.5×10^5 cells/well) and incubated at 20°C for 48 h until reaching 90-100% confluency (stabilization period). Every 7 days, cells were harvested, seeded into a new plate (1:3 cell suspension:media), and maintained under the same conditions for 36 days. Acute toxicity tests with 3,4-dichloroaniline (DCA), the OECD 249 positive control, assessed whether media composition influenced chemical response. Culture quality was evaluated through morphology and viability using

alamarBlue™ (metabolic activity), CFDA-AM (membrane integrity), and Neutral Red (NR, lysosomal integrity). The optimized culture condition was used to evaluate the performance of this *in vitro* model for chronic exposure assay with two cosmetic preservatives (benzyl alcohol (BA) and phenoxyethanol) and DCA, all with chronic fish toxicity data (NOEC). For the chronic exposure assay, cells were exposed to six concentrations of each substance defined based on EC20 from acute toxicity data (BA: 161.7-646.9 mg/L, phenoxyethanol: 0.07-66.8 mg/L, DCA: 1-2 mg/L), at 7-day intervals. After 28 days of exposure, cell viability was assessed using the indicator dyes, and NOEC values were statistically determined (ANOVA) using relative viabilities to the solvent control (DMSO). The L-15/ex, used for acute exposure, significantly reduces RTgill-W1 viability in long-term culture, emphasizing the need for a growth factor-enriched medium (e.g., FBS supplementation). L-15 with 1% FBS did not support proper cell attachment and growth over 36 days, whereas increasing FBS to 4.7% maintained cell viability without altering the response to chemical exposure (DCA). The optimized starting density of 2.5×10^5 cells/well with 1:3 reseeded ensured confluency within 48 h while preventing overgrowth during exposure. Not all viability indicator dyes were suitable for long-term culture, as NR staining caused significant cell detachment due to washing steps, compromising fluorescence measurements, while CFDA-AM exhibited poor reproducibility across experimental replicates. In contrast, alamarBlue™ proved to be the most reliable indicator dye for quantifying cell viability in long-term RTgill-W1 cultures. The chronic exposure assay determined a NOEC of 1.75 mg/L for DCA, closely matching the *in vivo* NOEC for *Danio rerio* (1.18 mg/L), demonstrating strong correlation between *in vitro* and *in vivo* chronic fish toxicity. Phenoxyethanol showed an *in vitro* NOEC of 16.8 mg/L, aligning with the *in vivo* NOEC (24 mg/L, *Pimephales promelas*). For BA, preliminary results indicated a NOEC < 161.7 mg/L, requiring concentration range adjustments. Overall, the RTgill-W1 cell line cultured with L-15 + 4.7% FBS and alamarBlue™ as the viability dye shows promise for chronic fish toxicity testing (mortality), though further research is needed to assess its performance across more chemicals.

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Evaluation of buccal film performance through porcine mucosa using side-by-side diffusion cells

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Introduction: The use of antineoplastic chemotherapeutics for cancer treatment has become increasingly frequent. These medications cause nausea and vomiting as side effects and are considered the main cause of discontinuation of treatment. Therefore, the use of antiemetics together with chemotherapy can improve nausea and vomiting attacks in patients approximately 25% to 50%. These medications are generally administered either intravenously or orally. However, the use of the intravenous route, despite the rapid effect and 100% bioavailability, does not seem to be convenient for a fragile patient. An antiemetic being tested in the country, here coded as AE01, has bioavailability of approximately 60% after oral administration, undergoes hepatic first-pass effect and has a short elimination half-life. For that reason, frequent administration is necessary to maintain clinically effective plasma concentration. The oral mucosa, and especially the sublingual mucosa, which is significantly more permeable, has been widely used for transporting drugs aiming at systemic effects in a few minutes. Given the above, one of the strategies to overcome these route-related inconveniences is oral administration.

Objectives: To evaluate the transmucosal performance (porcine origin) of a sublingual film containing AE01.

Material and methods: Permeagear[®] side-bi-side horizontal diffusion cells with 0.78 cm² of diffusion area and 3.4 mL of donor and receptor compartment volume were used, with water being the receptor liquid used in this study. In the test using the film, the donor compartment was filled with 3.4 mL of artificial saliva pH 6.8, and for the solution (used as a control), it also filled the entire volume of the compartment. Films containing 5mg of AE01 and a 5 mg/mL solution of the active ingredient were tested. The membranes used were sublingual, jugal and esophageal from pigs, all dermatomized in 250 µm thickness (n = 6 for each experiment). The test was performed under controlled agitation and temperature (250 RPM and 37 ± 2°C), collection times of 0.25; 0.5; 0.75; 1.0; 1.25; 1.5; 1.75; 2 hours. Finally, the donor and recipient compartments and the amount of AE01 retained in the mucous membranes were quantified.

Results: The mass balance showed that practically all AE01 dosed was found in the donor compartment (residual formulation), regardless of whether the formulation applied was film or solution. That said and considering the amount of active ingredient retained and permeated through the mucous membranes, it was observed that, regardless of the type of mucosa, there was greater retention, approximately 1.2-2.0 times when applying the film formulation when compared to the solution, also leading to greater permeation of AE01 from the film formulations.

Conclusion: Despite the quantitative observation of low permeation of AE01, the permeation profile suggests a linear flow sufficient to discriminate differences between formulations (film and solution), where the film presented greater permeation of the active ingredient regardless of the mucosa used, suggesting that some excipient of the formulation when in contact with the mucosa favored the passage of the drug. The results also suggest that, for evaluating transmucosal passage, the use of side-bi-side cells appears to be a good alternative to performance tests using vertical cells (OECD TG 428).

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Toxicogenomic and computational strategies for profiling the safety of a botanical extract using next-generation risk assessment approach

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The growing demand for safe, sustainable, and scientifically validated cosmetic products has driven innovation in safety assessment methodologies. Consequently, the safety evaluation of raw materials has undergone a paradigm shift towards advanced alternatives, such as computational modeling and toxicogenomic strategies, aligned with next-generation risk assessment (NGRA) frameworks. Botanical extracts, widely utilized in cosmetic and pharmaceutical formulations, often have complex chemical compositions, making their evaluation particularly challenging. However, by integrating computational and toxicogenomic approaches, it becomes possible to systematically profile the safety of such ingredients while estimating their biological impact at cellular and molecular levels. This convergence of modeling tools enables the assessment of critical endpoints such as skin sensitization, irritation potential, and dose-dependent responses of several key events (KEs) at the molecular level of adverse outcome pathways (AOPs), while adhering to NGRA principles. In this study, the application of computational modeling and toxicogenomic strategies for the safety profiling of a botanical extract was explored. Using transcriptomic data and mathematical models, insights were generated on the relationship between the compound's biological activity and its safe use limits through physiologically-based pharmacokinetic (PBPK) modeling. The natural extract of Piper methysticum was evaluated for its four main compounds: desmethoxyyangonin, kavain, yangonin, and methylsticin. The hazard assessment of these substances was conducted using toxicogenomic information retrieved from CompTox, combined with the development of a toxicological prioritization index based on the concentration of each compound within the extract. In addition, the *in silico* tools were used to estimate the toxicity potential to cause dermal and ocular irritation, skin sensitization, and acute toxicity. In total, 13 genes (ESRRA, NFE2L2, PGR, NR1I2, NR1I3, HDAC1, ESR1, RARA, SHH, THRA, THRB, RAD54L, XRCC6P1) and 22 assays associated with AOPs and modes of action (MoAs) were evaluated for endpoints such as cancer, developmental and reproductive toxicology (DART), and acute toxicity. Based on these data, a calculation was performed to extrapolate the concentrations to *in vivo* conditions. From this analysis, a safety threshold was estimated by applying a safety factor to determine a dose at which no KE (key event) for the selected MoA would be activated. Subsequently, using the htk (high-throughput toxicokinetics) package, a multi-compartment PBPK model was implemented to predict the distribution of the compounds across the gut, artery, vein, lung, liver, kidney, and rest-of-body compartments following oral exposure. Through this framework, a safe dose of 2 mg/kg for the extract was established. This integrative approach not only enhances our understanding of the extract's mechanistic effects but also exemplifies the potential of NGRA to evaluate the safety assessment process for complex natural ingredients.

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Risk and efficacy assessment based on transcriptomic data of a microalgae extract through the expression profile of interleukin-related pathways

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The development of natural extracts for cosmetic applications has gained significant attention in recent years, driven by the growing demand for safe, sustainable, and effective products. In this context, compounds produced via biotechnological processes have proven to be an excellent alternative to accelerate the development of raw materials with cosmetic properties. Microalgae, for example, are frequently used in biotechnological production and are valuable sources of carotenoids, chlorophyll, natural pigments, and, which have the potential to enhance skin health and appearance. *In vitro* models, such as reconstructed skin, combined with genetic sequencing techniques (e.g., RNA-seq), serve as powerful tools for evaluating the safety and efficacy of raw materials under development. Thus, this study aimed to leverage transcriptomic data obtained through sequencing in a 3D *in vitro* skin model to assess the efficacy of an algae extract (BAC: Bioactive Algal Compound) as a cosmetic ingredient and to estimate safe application doses based on the expression of genes related to interleukin pathways. The 3D skin models were treated for 24 hours with BAC at concentrations of 1%, 3%, and 5%. After the treatment period, the samples underwent RNA-seq analysis. Compared to the control group, a total of 434, 620, and 534 differentially expressed genes (DEGs) were identified for the respective concentrations of 1%, 3%, and 5%. For efficacy evaluation, the expression of over eight types of collagen was observed to increase by up to 193%, while hyaluronic acid production showed an increase up to 77-86%. Additionally, genes associated with the structural integrity of the epithelial barrier and skin firmness were positively modulated, indicating improvements in these skin parameters. To assess potential dermal sensitization and irritation, DEGs related to cytokine pathways were analyzed. A dose-response curve was created for these genes across the tested concentrations, using nine mathematical models to estimate benchmark dose (BMD), benchmark dose lower bound (BMDL), and benchmark dose upper bound (BMDU) values. This analysis demonstrated that the concentrations fall within safe limits for use, thereby ensuring the safety and efficacy of this raw material regarding skin sensitization and dermal irritation. However, additional experiments must be carried out to confirm these results. The findings suggest that the BAC extract shows great versatility for use in cosmetic products such as creams, serums, masks, and lotions. Moreover, preliminary studies indicate that the extract has a strong ability to stimulate collagen synthesis and reduce signs of aging through its properties that support the structural integrity of the skin, as well as its capacity to boost hyaluronic acid production.

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The critical role of a culture of care in supporting better science – For both people and animals

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A strong culture of care is essential in animal research, ensuring ethical, high-quality science that benefits both people and animals. Its four key pillars – animal welfare and the 3Rs, staff well-being, scientific quality, and openness – are deeply interconnected. Progress in one area reinforces the others, but fostering this culture requires institutional commitment, systematic implementation, and innovative approaches that challenge traditional norms. A culture of care naturally evolves into a culture of challenge, where critical thinking, continuous improvement, and innovation drive progress in research and animal care. Moving beyond compliance, this approach empowers staff to refine processes, embrace new methods, and proactively address challenges, leading to better animal welfare, stronger scientific outcomes, and a more engaged workforce.

This presentation explores strategies for embedding a culture of care, emphasizing its role in fostering innovation. Encouraging constructive questioning, evidence-based improvements, and transparent communication ensures sustainable progress. Compassionate leadership and staff empowerment cultivate resilience and engagement, enhancing research quality and workplace well-being. Findings from a longitudinal survey across five institutions that implemented compassion fatigue programs show that fostering compassion satisfaction improves staff retention, job satisfaction, and institutional stability. Support mechanisms such as peer groups and stress management training mitigate compassion fatigue and reinforce a positive, challenge-driven work culture.

Additionally, the talk highlights advancements in refined mouse handling techniques, which enhance both animal welfare and research reproducibility. Studies show that tunnel and cup handling, instead of tail handling, reduce stress responses in mice and improve behavioral data reliability. Adoption of these techniques benefits both animals and research outcomes by minimizing stress-induced variability, further demonstrating an institution's commitment to the 3Rs. To drive meaningful change, institutions must encourage employees to challenge existing practices and refine methods continuously. This requires leadership support, structured training, and a mindset that prioritizes ethics alongside scientific rigor. By embedding innovation and a commitment to challenge, organizations can transform their culture of care into a culture of challenge, creating an environment where both people and animals thrive.

This talk concludes with actionable recommendations for institutions seeking to enhance their culture of care while fostering a culture of challenge. Strategies include policy development, staff support mechanisms, and collaboration across scientific, veterinary, and husbandry teams. By embracing these approaches, organizations can continuously advance excellence in animal research and care.

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Using iPSCs to model the combined effects of genetics and aging on the human blood-brain barrier

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In addition to the ethical considerations of using animals for biomedical research, their high costs, low throughput, and limited translation motivates their reduction and replacement with human tissue-on-chip (hToC) models for disease modeling, drug development, and discovery. One frontier in hToC development is the modeling of aging as a confounding factor in disease. Our focus is on the blood-brain barrier (BBB), which is known to become less robust at regulating the passage of cells and molecules from blood as people age. Age-related changes in the BBB include cellular and transport changes that make the brain more vulnerable to injurious circulating factors and neurodegeneration. This includes impairment of lipoprotein receptor related protein-1 (LRP-1), which leads to amyloid beta (A β) accumulation in the brain, a hallmark of Alzheimer's disease (AD). In addition, the presence of the Apoe4 allele, a risk factor for AD, accelerates most of the aging related changes in the BBB. The mouse, a cost effective and common animal model, does not naturally develop human neurodegenerative diseases, including AD, and must be genetically engineered to approximate the condition. In animals that do naturally develop AD or cognitive decline such as non-human-primates (NHPs) or canines, the age-of-onset is too long for practical progress. Here, we leverage the use of small molecule drug cocktails to develop the first aged model of the blood brain barrier, the μ SiM-aBBB (microphysiological system enabled by a silicon membrane-aged BBB). We model geriatric vulnerabilities in combination with genetic predisposition of the Apoe4 allele. Specifically, we are mirroring the cellular changes that occur during aging including the induction of senescence, shifts in protein expression and increases in BBB permeability. Consistent with the onset of AD occurring in older patients homozygous for Apoe4, we show that aged cellular phenotypes combined with the Apoe4 mutation to produce a BBB that is intrinsically compromised compared to Apoe4 or aging alone. This novel tool can be used to determine ways to prevent barrier dysfunction under compromised conditions. This project will demonstrate the value of hToC models to emulate human health and disease.

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Dermal dressing developed with a natural resin *sangue de bicaíba*: A preliminary *in vitro* assay

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Radiodermatitis is the main complication of radiotherapy, which in more severe cases turns into radionecrosis. To improve the life quality of radiotherapy patients, the aim of this study is the application of the resin *sangue de bicaíba* in the development of a dermal dressing for the prevention of radiodermatitis. We will use this resin extracted from the native tree species of the Atlantic Forest *Virola bicaíbya* as the main component of the biomaterial proposed for its powerful biological effects such as antioxidant, wound healing and anti-inflammatory. For years this plant has been used and known by folk medicine. According to ISO 10,993, the first biocompatibility test to be carried out to evaluate the safety of biomaterials is the cytotoxicity assay. To perform the cytotoxicity assay, some solvents were tested to dissolve the resin: water, ethanol, methanol, butanol, ethyl acetate and propanone. The solvents were tested in 1:10, 1:1 and 10:1 proportion. Once the best solvent was found, a mother solution of 100 mg/mL of resin was prepared and a serial dilution was made in culture medium. Cytotoxicity assay was evaluated in a 96-well microplate containing 10,000 fibroblasts NIH-3T3 (ATCC Number: CRL-1658) cells/well. We incubated the fibroblasts for 24 hours with a serial dilution of resin, vehicle and positive control (DMSO), as negative control just culture medium was used. After that, the culture medium with solutions was removed and the 96-well microplate was incubated for another 3-4 hours with MTS/PMS dye and analyzed by spectrophotometer at 490 nm. Water was the best solvent because dissolved better and when the mother solution was diluted in culture medium it was not precipitated. The vehicle (water) used to dilute the resin did not show cytotoxicity at any of the concentrations used, which is very important because the solvent used did not interfere with the cytotoxicity of the resin. Others biocompatibility tests indicated for ISO 10,993 to evaluate the safety of the proposed biomaterial are the irritation and sensitization assays that will be made with resin concentrations above 90% of cell viability found in cytotoxicity assay (0.74 mg/mL). We believe in the radioprotective potential of the resin *sangue de bicaíba* since it combines the antioxidant and anti-inflammatory effects with the wound healing capacity. Based on these preliminary results of the cytotoxicity assay, the resin *sangue de bicaíba* has great potential to be the active ingredient for the development of the dermo dressing in order to prevent radiodermatitis, helping not only in the life quality of radiotherapy patients, but also allowing the continuity and completion of the treatment.

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Development of a synergistic approach to explore the potential of natural compounds as anti-aging active ingredients by protease inhibition

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The growing demand for clean beauty cosmetics has driven companies to develop products with fewer ingredients and potent efficacy derived from natural sources, particularly in skincare targeting anti-aging properties. Natural extracts are a rich source of molecules with diverse functionalities, including the ability to modulate multiple biological pathways. Elastase (ELA) and Collagenase (MMP), enzymes responsible for degrading elastin and collagen during aging, play a significant role in remodeling the skin's extracellular matrix, leading to a loss of elasticity and reduced support in connective tissue. This makes ELA and MMP key targets for evaluating potential anti-aging compounds. This study aimed to develop a methodology to assess the anti-aging potential of two novel molecules, GBx1 and GBx2, using bioinformatics tools and *in chemico* validation. The bioinformatics analysis involved utilizing toxicogenomic databases to gather experimental data on genes modulated by these compounds. Subsequently, systems biology techniques were employed to identify activated biological processes and pathways. A total of 88 and 85 genes were identified for GBx1 and GBx2, respectively. Functional enrichment analysis revealed that some genes modulated by these compounds are involved in extracellular matrix remodeling and aging. Additionally, virtual screening techniques were used to computationally predict the efficacy of these molecules. Molecular docking was performed using a 3D protein model of ELA and MMP, and a ligand-based screening was conducted with natural ELA/MMP inhibitors. Docking results indicated strong binding affinities for GBx1 and GBx2, with up to 72% structural similarity to natural inhibitors. Based on the computational characterization of these molecules to inhibit ELA and MMP activity, an *in chemico* assay was performed to experimentally validate the bioinformatics predictions. Seven concentrations of each molecule were tested, along with a positive control. GBx1, at the tested concentrations, did not inhibit ELA activity. However, GBx2 demonstrated significant inhibitory potential with an IC₅₀ of 0.04 mg/mL. Similar results were observed for MMP inhibition. Additionally, a synergy study was carried out by mixing all tested concentrations, and the results were evaluated using mathematical models. The concentration that showed the highest synergy, as validated by statistical modeling, was selected and applied to *in vitro* reconstructed models for safety evaluation (OECD 439 and 492). Furthermore, the potential efficacy of these molecules across various biological pathways was elucidated through RNA-seq analysis using a 3D skin model and immunohistochemistry. In conclusion, the synergistic integration of bioinformatics assessments and *in chemico* testing establishes a robust approach for



the development of natural anti-aging ingredients. This strategy offers a promising pathway for further investigations into the efficacy of natural compounds in modulating the aging process and promoting skin health.

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A bioinformatics framework for unveiling hair growth regulatory networks for evaluation of raw materials and cosmetic efficacy

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The continuous development of the cosmetic industry promotes the stimulation and search for new biomarkers capable of evaluating the effectiveness of products. In this context, the use of integrative methodologies has become an important topic in the industry. Bioinformatics tools offer a crucial solution, enabling the integration of data from diverse experiments and providing comprehensive analyses of gene expression profiles. This allows for the identification of genes and pathways relevant to specific biological processes, enabling the development of effective cosmetics. This study addressed the knowledge gap regarding the effects of various cosmetic compounds on hair growth by integrating transcriptomic data from microarray datasets of human hair follicle dermal papilla cells (HFDP cells) exposed to hair growth stimulants. Datasets GSE178510, GSE163091, GSE199648, GSE199651, and GSE211839, obtained from the NCBI-GEO database, comprised *in vitro* experiments where HFDP cells were treated with multiple hair growth stimulants. Using the R package bioconductor – limma, the datasets were analyzed to compare experimental conditions. Volcano plots and Venn diagrams were employed to identify genes with significant ($\text{padj.} < 0.05$) differential expression. Functional enrichment analyses were then performed to explore associated biological pathways and processes. The transcriptome of HFDP cells exposed to the chemicals revealed 1393 commonly differentially expressed genes across all datasets. The majority of these genes were linked to biological processes related to cell differentiation, proliferation, and increased protein synthesis. Further analysis focused on the enriched phenotype “hair morphology measurement,” containing 72 genes. All tested compounds activated pathways regulated by 9 main key regulators genes. This comprehensive analysis elucidated the biological pathways regulated by these genes, providing insights into the mechanisms by which hair growth stimulant raw materials function. Based on these findings, the evaluated genes can be considered as potential biomarkers for assessing the efficacy of raw materials, active ingredients, and hair care products designed to promote hair growth. This research offers a valuable resource for the development of more effective and targeted hair care products and contributes to the advancement of animal-free testing methodologies in the cosmetics industry.

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Promoting just laws and the public good – The US HEARTS Act and the American Bar Association resolution on animal testing

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Non-animal research methods are increasingly benefiting many areas of biomedical research, including cancer, diabetes, cardiovascular disease, Alzheimer’s disease, mental illness, infectious disease and rare diseases. Still more new approaches are needed to further advance the 3Rs and to address public health and medical challenges. The National Institutes of Health (NIH) is the world’s largest funder of biomedical research in the world and much of this research is animal-based. Meanwhile there is widespread support amongst US taxpayers (the source of NIH funding) to move away from animal-based research and to prioritize non-animal methods.

While US law is based on the principles of the 3Rs, there is no legal obligation to apply any of them. The Animal Welfare Act (Public Law 89-544) requires researchers to consider alternatives to animal use for painful procedures and stresses that researchers should not unnecessarily duplicate previous experiments. However, oversight is lacking, and these provisions are not implemented the way that Congress intended. The bipartisan Humane and Existing Alternatives in Research and Testing Sciences (HEARTS) Act targets specific shortcomings in existing law that govern NIH-funded research proposals. The Act would establish a dedicated center within the NIH for the development of non-animal methods and collection of data on animal use.

In early 2024, the American Bar Association’s House of Delegates passed Resolution 502, urging national governments, U.S. Congress, and federal agencies to support the development and implementation of methods aimed at achieving the 3 Rs with emphasis on replacement. The resolution report focused on removing barriers to and creating incentives for the use of nonanimal research and testing methods in regulatory and federally sponsored research. The resolution highlighted the HEARTS Act as legislation that could help achieve this goal and concluded that, “the replacement of animal-based research and testing with scientifically suitable, cost-effective and humane nonanimal methods promotes just laws and the public good.”

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Role of non-animal methods in delivering the UN 2030 agenda for sustainable development

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This poster will provide an overview of the 17 UN Sustainable Development Goals (SDGs) and how development and uptake of nonanimal methods can aid in reaching some of those goals.

The Sustainable Development Goals (SDGs) are a set of 17 non-legally binding, global goals adopted by the United Nations (UN) General Assembly in September 2015 as a core part of the 2030 Agenda for Sustainable Development. The 17 goals are accompanied by 169 targets that are aimed at ending poverty, protecting the planet improving equity to meet the needs of the most disadvantaged people and ending discrimination against women and girls.

Although animal welfare is not explicitly listed as one of the 17 Sustainable Development Goals, many of the targets within these goals focus on biodiversity, species protection, and habitat conservation. The NGO Major Group, one of the nine “Major Groups” established by the UN, plays a key role in facilitating civil society participation in discussions and decision-making on sustainable development. This group is organized into thematic clusters of issue experts, one of which is the “Animal Issues Thematic Cluster” (ACTIC). In March 2022, during the fifth session of the United Nations Environment Assembly (UNEA-5), the first resolution directly addressing animal welfare was adopted – Resolution 5.2/1, titled “Animal Welfare, Environment, and Sustainable Development Nexus.” This resolution highlights the interconnection between animal health and welfare, sustainable development, the environment, and human well-being. It also recognizes that improving animal welfare can help tackle environmental challenges, support the “One Health” approach, and contribute to the achievement of the Sustainable Development Goals.

Twenty-first century scientific and technological advancements will be crucial in meeting many of the SDGs. Modern human-based approaches promise to deliver new medicines, vaccines, and safer chemicals that can better protect human health and the environment and minimize harm to animals. Moreover, ensuring that the benefits of and access to the new methodologies are shared globally will also play a major role in meeting the SDGs.

While non-animal methods have the potential to play a role in many of the SDG the replacement of animals in testing is arguably most relevant to Goals 3 (Good Health and Well-Being), 12 (Responsible Consumption and Production), and 17 (Partnerships for the Goals).

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Rotifers as an alternative experimental model of estrogenicity

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Any substance capable of interacting with the estrogen receptor is considered to exhibit estrogenic activity. Estrogenicity represents a toxic mode of action, as it can mediate chronic effects such as metabolic disorders, cancer, and reproductive abnormalities. Estrogenic effects are traditionally assessed using the rodent uterotrophic assay, which relies on female rats to evaluate uterine weight increases as a response to estrogenic stimulation. In this study, we propose the use of *Brachionus* spp. rotifers, aquatic invertebrates, as an alternative experimental model for evaluating estrogenicity. Acute 48-hour toxicity assays were conducted to determine the median lethal concentration (LC50) of test substances in the presence and absence of the estrogen receptor antagonists tamoxifen and its active metabolite, 4-hydroxytamoxifen. The addition of these antagonists caused a significant shift in the dose-response curve, increasing LC50 values for both substances. This shift suggests that estrogenic responses are conserved between rotifers and vertebrates, supporting the potential use of these organisms as an alternative model for studying estrogenic effects. Rotifers in laboratory cultures predominantly exist as clonal female populations, which reduces response variability. Moreover, these microscopic invertebrates lack a complex nervous system and are not considered conscious or sentient, addressing ethical concerns associated with vertebrate testing. These characteristics support the feasibility of using *Brachionus* spp. as a model for assessing estrogenic effects in invertebrates and lay the groundwork for further validation of aquatic animal models in the study of estrogenic endocrine disruption.

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Initial approach to the protein profile associated with the skin mucosa of *Hoplias malabaricus*

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Continuous monitoring of aquatic environments is essential for assessing their health status. Due to both biotic and abiotic factors – including overexploitation, pollution, habitat loss, and fluctuations in water temperature – valuable aquatic resources are becoming increasingly vulnerable to both natural and anthropogenic environmental changes, potentially affecting their physiology. Understanding fish health and well-being is crucial for preserving species diversity. The use of species closely associated with the studied environment is a key strategy; however, it is essential to adopt non-invasive methods that minimize ecosystem impact and reduce the need for animal sacrifice. Since fish are in constant contact with their surroundings, skin mucus is considered a first line of defense against a wide range of environmental conditions. Mucosal surfaces, particularly the skin, play a critical role in maintaining body integrity, homeostasis, and preventing pathogenic skin infections. In recent years, proteomic techniques have gained increasing interest in fish biology research. Proteomics has primarily been employed in studies related to physiology, developmental biology, and the impact of pollutants on fish. However, its application has been mostly

limited to a few model organisms, such as zebrafish (*Danio rerio*) and certain salmonids. The limited genetic information available for most fish species remains a major challenge, restricting the broader application of proteomic technologies. Considering these factors, we propose skin mucus analysis as a non-invasive and reliable method for studying the physiological response of *Hoplias malabaricus* and identifying proteins that may serve as biomarkers or indicators of environmental challenges and stressors.

Methodology: For this study, wild *Hoplias malabaricus* specimens were collected and anesthetized using 300 ppm of 2-phenoxyethanol. Mucus samples were obtained from the midline using gentle scraping with sterile sponges, then transferred to reaction tubes and preserved in liquid nitrogen until analysis. The samples were lysed in the presence of 2% sodium deoxycholate, followed by reduction and alkylation processes. Subsequently, enzymatic digestion with trypsin was performed, desalting was carried out using C18 columns, and the resulting tryptic digests were analyzed via LC-MS/MS. To gain deeper insights into the biological relevance of the expressed proteins, bioinformatics analysis with functional enrichment was conducted.

Results: An initial characterization of the proteomic profile of *H. malabaricus* mucus was performed based on functional homology, identifying proteins associated with three main functions: structural, metabolic, and protective Conclusion. This study analyzed the skin mucus of *H. malabaricus* as a potential non-invasive tool for assessing fish health, extending beyond its defensive capabilities.

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Recommendations for addressing pharmaceutical sector-specific challenges to meet the European Commission's goal of phasing out animal testing for regulatory purposes

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The European Commission is developing a roadmap to phase out animal testing for chemical safety assessments, set for publication in 2026. Human medicines are included in the broad definition of chemicals subject to this roadmap. However, while regulatory requirements overlap across chemical sectors, differences in data requirements and review procedures warrant careful consideration. This poster outlines the nuances of different regulations, identifies pharmaceutical sector-specific challenges, and recommends concrete actions to integrate the pharmaceutical sector into the broader roadmap.

Notably, this roadmap is being developed alongside a major revision of EU legislation for medicines for human use, initiated by the 2020 EU Pharmaceutical Strategy. A key objective of this revision is to foster an innovation-friendly environment for the research, development, and production of medicines in Europe. The Commission's roadmap for phasing out regulatory animal testing represents a crucial opportunity to support the implementation of innovative methods for testing the efficacy, safety, and quality of pharmaceutical products.

To support alignment between the roadmap and revision of the pharmaceutical legislation, this poster identifies ten solutions for challenges across five key topics: research collaboration and incentives, data sharing, data requirements, regulatory frameworks and policies, and public engagement. Implementation recommendations target key stakeholders, including the European Commission, the European Medicines Agency (EMA), national and international regulators, the pharmaceutical industry, academic researchers and non-governmental organizations.

The proposed recommendations range from immediate administrative steps – such as fee waivers for scientific advice on non-animal testing strategies and increased funding for EU consortia projects designed to fill methodological gaps – to more complex actions, including the establishment of a test guideline program and a Board of Appeal at the EMA requiring broader stakeholder involvement. Ensuring the roadmap effectively addresses pharmaceutical sector needs will maximize opportunities for replacing tests on animals that might be otherwise overlooked under a one-size-fits-all approach to chemical regulation.

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Infection of liver 3D spheroids with yellow fever virus: A novel experimental approach

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Yellow fever is a viral disease transmitted by mosquitoes in tropical endemic regions such as Africa, Central America, and South America. The disease primarily affects the liver, causing varying degrees of hepatic damage. In unvaccinated individuals, the disease can progress rapidly to severe forms. Given the lack of specific treatment, vaccination remains the primary preventive strategy. Consequently, understanding the pathophysiology of the disease *in vitro* models is crucial. In this study, we developed hepatic 3D spheroids (C3A:HHStC cells) as Yellow Fever Virus (YFV) infection model. Spheroids were infected with a multiplicity of infection (MOI) of 1.0 using the vaccine strain of YFV and incubated for four days. Cell viability, assessed by the MTT assay, showed reduction in YFV-infected spheroids viability ($45\% \pm 5.8$) compared to control ($100\% \pm 33.46$). Structural analysis using scanning electron microscopy (SEM) revealed an altered spheroid morphology upon infection and a decrease in spheroid size. Scanning transmission electron microscopy (STEM) provided ultrastructural insights, showing organelle integrity loss in infected spheroids. Immunofluorescence staining with 4G2 confirmed successful flavivirus infection. Immunofluorescence analysis revealed reduced marker of Cytokeratin 19, Albumin, and CYP3A4/5, indicating impaired hepatic function. qPCR analysis corroborated these findings, showing a significant reduction in albumin (control 1.15 ± 0.45 ; YFV 0.28 ± 0.01) and CYP3A4 expression (control 0.99 ± 0.30 ; YFV 0.47 ± 0.10). Furthermore, cytokine quantification revealed increased production of TNF α , IL-1, and IFN α , signaling a robust inflammatory response. Mass spectrometry (LC-MS/MS) identified viral proteins, including NS1, NS3, and NS5, and revealed modulation of innate immunity and antiviral defense pathways. Increased levels of proteins such as SODM, TBB5, and ARPC3 were associated with the activation of an inflammatory response, reflecting the host's immune reaction to infection. These findings confirm that YFV infection leads to significant loss of hepatic function in spheroids, with activation of inflammatory and antiviral pathways. These effects closely resemble those observed *in vivo*, underscoring the importance of 3D models for viral infection in preclinical studies, reducing reliance of experimental animals.

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U-SENS: A game-changer for skin sensitization assessment under OECD TG 497

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Skin sensitization, an adverse reaction to repeated skin contact, is a key safety concern for cosmetics. Animal models such as the LLNA have historically been used to assess this, but ethical and regulatory pressures favor alternative *in vitro* and *in silico* approaches. To address this, promising OECD-adopted Integrated Testing Strategies (ITS V1, ITS V2) combine *in chemico* assays for protein binding (KE1), *in vitro* methods for dendritic cell activation (KE3), and *in silico* predictions (OECD GL 467). These ITS aim for reliable potency categorization, aligning with the Globally Harmonized System (GHS) while minimizing animal reliance (UN GHS Cat. 1A, 1B, NC).

The current work outlines an ITS DA using the Direct Peptide Reactivity Assay (DPRA) and the U-SENS assay, alongside *in silico* tools like Derek Nexus (ITS V1) or the OECD QSAR Toolbox (ITS V2). The ITS DA is broken down into different scenarios, looking at 4 types of profiling with the combined *in chemico/in vitro* assays and *in silico* data. The results are translated into a scoring system, culminating in a total battery score that predicts both hazard identification (UN GHS Cat. 1 vs. NC) and potency sub-categorization (UN GHS Cat. 1A, 1B, NC). The performance of the ITS DA was assessed against reference datasets including 168 Local Lymph Node Assay (LLNA) data and 66 human data.

Results showed hazard identification performance and demonstrated accuracies in the range of 86% for LLNA and 88-86% human data. The potency sub-categorization showed a similar balanced accuracy of 79% tested against LLNA Potency and 73-74% tested against human data for potency as well, demonstrating comparable accuracy relative to the LLNA. Notably, when only an *in-silico* tool is associated with one KE assay only (either DPRA or U-SENS), no ingredient is categorized as NC, being side protective to encourage to perform the second assay to reach potency sub-categorization.

Overall, the ITS strategy, that uses a combination of Derek/Toolbox – DPRA – U-SENS provide an accurate and reliable assessment of skin sensitization potential. These findings support the inclusion of the strategy with the alternate U-SENS™ assay in guideline 497.

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Shaping the future of sepsis research with human-based models

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Sepsis, a life-threatening condition resulting from the body's response to infection, affects millions globally every year, leading to significant mortality and economic burden. Despite its prevalence, advancements in sepsis therapies have been hindered, in part, by the poor translation of findings from animal models, particularly mice. This literature review examines the limitations of commonly used animal models of sepsis and emerging, human-based models. A comprehensive literature review was conducted using PubMed to obtain studies published within the past five years. The search was further refined using the terms “*in vitro*,” “*in silico*,” “computational,” and “mathematical” to identify studies focused on basic and mechanistic sepsis research. Studies using live non-human animals, cells/tissues from non-human animals, non-human biology models, case reports, meta-analyses, clinical trial protocols, non-English articles, and studies unrelated to sepsis or sepsis-causing pathogens were excluded. Mice are commonly used in sepsis research due to their low cost and ease of use; however, mounting evidence suggests that their genetic and physiological responses to sepsis differ markedly from those of humans, impeding the development of effective therapies. Human cell culture techniques, computational modeling, and other methods have advanced significantly over the past decade and offer promising avenues for enhanced translation. Studies using human cell culture techniques, particularly those utilizing three-dimensional (3D) culture models, offer a closer approximation of human tissue morphology and function. These 3D models provide a more accurate representation of disease mechanisms such as host-pathogen interactions and sepsis progression, which are critical for identifying effective therapeutic targets. Additionally, studies that employ *in vitro* models allow for a more focused investigation of specific aspects of sepsis pathophysiology and the integration of genomic data from human patients, including personal genomes and exomes, further strengthens the ability of these models to reflect human disease heterogeneity. Human biology-based approaches align more closely with the complex nature of human sepsis and many have the potential to replace animal models.

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***In silico* evaluation of the pulmonary toxicity of a pharmaceutical compound and perspectives for organ-on-a-chip studies**

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Introduction: Toxicological assessment is crucial in drug development to ensure safety before clinical application. Traditional *in vivo* tests using animal models present ethical and scientific challenges, reinforcing the need for alternative methods. *In silico* models predict compound toxicity based on structural rules and chemical data, while organ-on-a-chip (OOC) models have emerged as innovative tools that simulate physiological microenvironments in microfluidic devices containing human- and animal-derived cells. Integrating *in silico* and OOC approaches holds great potential for optimizing toxicological evaluations while reducing reliance on animal testing.

Objective: To describe findings from an *in silico* tool predicting potential toxic effects of a pharmaceutical compound intended for use in bovines and discuss experimental validation perspectives using a lung OOC model.

Methodology: An SAR-based (Structure-Activity Relationship) software tool was used to qualitatively assess the relationship between the compound's chemical structure and its biological activity. The analysis

relied on integrated databases containing structural analogs and their associated biological effects in mammals.

Results: The *in silico* model identified a pulmonary toxicity alert (plausible) linked to the aromatic group specificity, which is often associated with pulmonary edema and pleural effusion. The alert's reliability ranged from moderate to high (40-70%), with an experimental concordance rate of 50-80%. Metabolic bioactivation may generate reactive metabolites, increasing vascular permeability and damaging pulmonary endothelial cells.

Given the moderate to high probability of experimental confirmation, a bovine-specific lung OOC model could be developed to validate these *in silico* findings. OOC models exhibit 80-95% sensitivity and 70-90% specificity. However, developing a pulmonary OOC model for bovines requires adapting cell culture protocols to replicate the species' physiological conditions. The incorporation of pulmonary metabolic enzymes could enable metabolite conversion analysis, identifying reactive species responsible for endothelial damage. Additionally, the inclusion of immune cells (macrophages and neutrophils) would allow the assessment of inflammatory mediator release and oxidative stress, both of which are key factors in chronic inflammation and lung injury. Further investigation into metabolic pathway interference could provide deeper insights into the role of bioactivation in progressive tissue damage.

Conclusion: The results highlight the need for further investigations to validate the predicted toxicity and elucidate underlying mechanisms. The next phase will involve exposing the lung OOC model to the compound for in-depth evaluations. These findings reinforce the importance of integrating *in silico* and OOC models in drug development, improving toxicological assessments, reducing animal testing, and providing more precise insights into adverse effects, ultimately aiding in the development of safer and more effective therapies.

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Development of an *in vitro* model to assess ocular damage and reversibility using bovine limbal mesenchymal stromal cells

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Assessing ocular toxicity of chemical products is essential globally. The Draize rabbit test, a historical standard for chemical classification under the UN Global Harmonized System (GHS), faces ethical and scientific limitations, prompting efforts to develop *in vitro* models. Current validated methods, such as the Bovine Corneal Opacity and Permeability (BCOP) assay, are effective at identifying non-irritants and severe damage but fail to assess moderate, reversible effects – a critical limitation for determining GHS Category 2 classifications and understanding wound healing.

Limbal mesenchymal stromal cells (LMSCs) play a critical role in the corneal wound-healing process, making them particularly relevant for evaluating recovery after chemical exposure. In this study, we isolated LMSCs from bovine corneas, the same tissue used in the validated BCOP assay. This substrate allows us to expand upon the BCOP's established role in ocular toxicity testing while addressing its key limitations in assessing damage reversibility and healing.

Isolated bovine LMSCs expressed surface markers CD73, CD90, CD105, CD29, and CD39, showed no expression of CD45 or HLA-DR, and had a low percentage of cells expressing CD34. Immunofluorescence analysis revealed expression of SOX2 and ABCG2, but no CK3 or CK12, aligning with literature data. To determine whether bovine LMSCs could effectively predict ocular toxicity in the human cornea, a cytotoxicity assay was performed to compare human and bovine LMSC sensibility to four reference chemicals. The effects of ethanol, benzalkonium chloride (BZK), Triton X-100, and sodium lauryl sulfate (SLS) showed a dose-dependent reduction in cell viability for both bovine and human LMSCs. Human LMSCs exhibited greater sensitivity to SLS and higher resistance to Triton X-100, while both cell types responded similarly to ethanol and BZK.

To simulate real-world accidental chemical exposure and assess the potential for subsequent healing, a scratch assay was used to evaluate the effects of short-term (5-minute) treatments with various reference substances at three different concentrations. No Category chemicals exhibited migration rates comparable to the control across all concentrations, while intermediate category chemicals only inhibited wound closure at higher concentrations. Category 1 chemicals completely prevented wound closure at all concentrations due to cell viability loss. However, concentration curves for Category 2 chemicals (isopropanol and ethanol) showed no significant differences in migration rates when cells remained viable after exposure; migration rates either were similar to the control or cell death occurred.

In conclusion, this study introduces a novel approach to evaluating ocular toxicity and wound healing by using bovine LMSCs. The exploration of transient exposure in a scratch assay, which mimics real-world accidental chemical contact, highlights the importance of understanding brief exposure events and their impact on corneal wound healing.

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Cyto-Safe: Advancing early-stage cytotoxicity prediction in drug discovery via interpretable machine learning

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Cytotoxicity assessment is a critical step in early drug discovery to mitigate toxicological risks and reduce costly late-stage failures [1]. Traditional *in vitro* assays, while effective, face scalability and ethical challenges. To address this, we present Cyto-Safe, an open-access machine learning (ML) tool for predicting cytotoxicity using Quantitative Structure-Activity Relationship (QSAR) models [2]. Cyto-Safe employs a curated dataset of approximately 90,000 compounds tested on 3T3 (mouse fibroblast) and HEK 293 (human embryonic kidney) cell lines using the CellTiter-Glo assay. The approach utilizes Extended Connectivity Fingerprints (ECFP4) together with Light Gradient Boosting Machine (LightGBM) algorithms to classify compounds as cytotoxic or non-cytotoxic. To address class imbalance, NearMiss v.3 under-sampling was applied, resulting in balanced accuracy (BACC) values of 0.92 for 3T3 and 0.90 for HEK 293 at a 1:5 ratio. The models demonstrated robust performance, with Matthews Correlation Coefficients (MCC) of 0.88 for 3T3 and 0.84 for HEK 293. Furthermore, Y-randomization confirmed the model's validity by yielding MCC values near zero (0.01-0.02), indicating the absence of random correlations. Cyto-Safe's web interface (<http://insightai.labmol.com.br/>) al-

lows users to input SMILES strings, sketch molecules, or upload CSV/SDF files. Predictions include binary outcomes, confidence scores, and explainable AI (XAI) visualizations, highlighting structural fragments influencing toxicity via color-coded heatmaps. The tool adheres to OECD guidelines [3], with applicability domain analysis ensuring reliability. Comparative analysis with existing tools underscores Cyto-Safe's strengths: interpretability, accessibility, and no installation requirements. Beyond its high predictive performance, Cyto-Safe's XAI framework enhances model transparency, allowing researchers to visualize the molecular features driving toxicity predictions. The tool facilitates virtual screening campaigns by identifying compounds with lower cytotoxic risks, thus optimizing candidate selection for further experimental validation. The tool is built on open-source frameworks, ensuring transparency and reproducibility, with model training protocols and datasets publicly available. Limitations include cell line specificity and assay-dependent generalizability. Future updates will expand to additional cell lines and assays, improving robustness and applicability across broader biological contexts. By fostering AI-driven toxicity prediction, Cyto-Safe contributes to safer drug development and aligns with ethical and technological advances in toxicology research.

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***In silico* comparative analysis of wound healing proteins: Evaluating the bovine model for ocular toxicity studies**

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The registration of chemicals requires evidence of potential eye damage. To meet this requirement, the Draize test performed in rabbits classifies chemicals into four categories based on lesion severity and healing time. However, alternative methods currently available do not assess damage reversibility or the time needed for recovery, as required by UN GHS classifications. Therefore, new approaches are needed to address this gap.

Although human-based models are the preferred choice for toxicity testing, access to human corneas for such assays remains limited. As an alternative, *ex vivo* animal tissues, such as bovine corneas from the meat industry, provide a more accessible option. This study conducts an *in silico* analysis of key wound-healing proteins across species to identify those most similar to humans, with a particular focus on assessing the bovine model's potential for evaluating corneal damage recovery.

A systematic bioinformatics approach was employed to compare the homology and structural similarity of wound healing-related proteins among *Homo sapiens*, *Pan paniscus* (bonobo), *Bos taurus* (bovine), *Oryctolagus cuniculus* (rabbit), and *Danio rerio* (zebrafish). Proteins relevant to wound healing were identified using the AmiGO 2 Gene Ontology (GO) database, focusing on connective tissue replacement (GO:0002248) and epithelial proliferation in wound healing (GO:0060054). The dataset was complemented with literature-reported proteins. Protein sequences were retrieved from UniProtKB, ensuring selection criteria included experimentally validated or high-confidence homologous sequences. Pairwise sequence alignments were performed using Clustal Omega (1.2.4) to determine percentage identity with human proteins. Physicochemical properties of selected proteins, including isoelectric point (pI), instability index (II), aliphatic index (AI), and grand average of hydropathy (GRAVY), were predicted using the ProtParam tool from ExPASy. Differences between species and humans were quantified, and a normalized score was generated to assess overall structural similarity.

The comparative analysis revealed that bovine proteins analyzed in this strategy exhibited higher sequence identity and structural similarity to human wound healing proteins than those from the rabbit model. Despite the rabbit being the gold standard for ocular wound healing studies, our results suggest that the bovine model may provide relevant biological insights. This study supports the rationale for using the bovine model in corneal wound healing research due to its molecular similarity to humans. These findings highlight the importance of *in silico* analyses in refining animal model selection and advancing the 3R principles (Replacement, Reduction, and Refinement).

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The impact of environmental tobacco smoke exposure on gestational diabetes mellitus severity: A public health perspective

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Background: Environmental tobacco smoke (ETS) is a major source of indoor air pollution and a significant public health concern, particularly for pregnant women and fetal development. While active smoking during pregnancy has been linked to adverse maternal outcomes, the effects of passive exposure to tobacco smoke on gestational diabetes mellitus (GDM) severity remain underexplored. This study investigates the association between ETS exposure, including secondhand smoke – and GDM severity, emphasizing the need for stronger public health interventions to protect maternal health.

Methods: This study utilized data from the Korean Pregnancy Outcome Study (KPOS), a prospective cohort of 3,457 singleton pregnancies, excluding pre-existing diabetes cases. GDM was diagnosed in 231 participants (6.7%), categorized into A1GDM (n = 198, 85.7%) and A2GDM (n = 33, 14.3%). Self-reported smoking behaviors, including active smoking before and during pregnancy, smoking intensity (pack-years), and secondhand smoke exposure, were assessed via validated questionnaires. Multiple logistic regression models estimated adjusted odds ratios (aORs) and confidence intervals (CIs) for GDM risk, adjusting for sociodemographic factors and cardiovascular risk variables.

Results: Active smoking before pregnancy (aOR 3.831, 95% CI: 1.520-9.656) and during early pregnancy (aOR 10.004, 95% CI: 3.053-32.782) significantly increased the likelihood of severe GDM (A2GDM). Additionally, smoking intensity exceeding 4 pack-years was strongly associated with A2GDM (aOR 22.393, 95% CI: 7.327-68.440). Critically, secondhand smoke exposure among never-smokers was also linked to increased overall GDM risk (aOR 1.360, 95% CI: 1.001-1.849), demonstrating the broader environmental impact of tobacco exposure.

Conclusion: These findings highlight the detrimental effects of both active and passive tobacco smoke exposure on maternal health, particularly in increasing the severity of GDM. Given the significant contribution of secondhand smoke to indoor air pollution, targeted public health policies – such as stricter smoke-free regulations and improved indoor air quality measures – are essential to protecting maternal and fetal health. This study underscores the need for global action to mitigate the environmental health risks posed by tobacco smoke exposure in pregnancy.

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Developing a testing battery for developmental neurotoxicity mediated via disrupted thyroid hormone homeostasis

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Introduction: Maternal thyroid function is critical for prenatal neurodevelopment. Maternal thyroid dysfunction is associated with lower IQ and delayed motor development in children. Endocrine disrupting chemicals (EDCs) are associated with disruption of maternal thyroid function and neurodevelopmental effects during pregnancy. *In vivo* animal data has limited relevance for toxicity prediction in humans. Our aim is therefore to develop a human-based testing strategy to assess the safety of chemicals for developmental neurotoxicity mediated by disruption of thyroid hormone (TH) signaling.

Methods: Testing battery tier 1 consists of human cell lines representing neurons (SK-N-AS), oligodendrocytes (MO3.13) and astrocytes (H4). These were exposed to 12 different chemicals. T3 and T4 transport and deiodinase-mediated metabolism were tested using 1 nM radiolabeled TH. T3-receptor activation was measured by gene expression changes of the T3-responsive gene KLF9 with qPCR. Testing battery tier 2 entails the differentiation of human stem cells into neuron-astrocyte co-cultures in which the effects of the lead EDCs from tier 1 are tested. Effect of chemicals on T3-receptor response and neuronal differentiation was measured using qPCR on a wider array of genes.

Results: Silychristin inhibited TH uptake up to 50% in all cell models. Whole-cell metabolism showed inhibition of deiodinase type 3 by iopanoic acid in MO3.13 and SK-N-AS cells. T3-induced KLF9 expression decreased 38% by TRα1 antagonist NH-3, and TBBPA exposure resulted in a fold change increase of ~8. Inhibition of T3-transport by 10 μM silychristin reduced KLF9 expression by 39%. In tier 2, 10 days of TBBPA exposure increases KLF9 expression. Exposure to other EDCs like chlorpyrifos show effects on gene expression related to neuronal differentiation.

Conclusion: The selected cell lines are suitable models to test the effect of EDCs on TH transport, metabolism and receptor activation. Human stem cells are a promising model to study the effect of EDCs on neuronal differentiation. Together, these models can form a two-tier testing battery to assess whether EDCs can disrupt the TH balance in the developing brain.

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Assessing neurodegenerative potential *in vitro*: The importance of timing, duration and frequency of exposure

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The neurodegenerative potential of pesticides cannot be captured adequately in current risk and safety assessment studies. As pesticide exposure is associated with an increased risk of developing Parkinson's disease (PD) later in life, there is a need for a novel risk and safety assessment framework for new substances entering the market. Here, standard acute toxicity tests may not suffice, given the long period of time for PD to develop and the diversity in real-life exposure. Our aim was to determine to what extent timing, duration and frequency of exposure affect pesticide-induced effects related to neurodegeneration *in vitro*.

To this end, neuroblastoma SH-SY5Y cells, differentiated to a dopaminergic phenotype, were exposed to four pesticides (dinoseb, endosulfan, mancozeb, and rotenone). Cells were exposed for a) 24 hours continuously without recovery, b) 24 hours continuously with 18 hours recovery, c) 24 hours continuously with 72 hours recovery, d) 24 hours divided over four days, or e) for 96 hours continuously. After exposure, mitochondrial metabolic activity and cytotoxicity were determined by alamar blue and lactate dehydrogenase assay, respectively. Internal pesticide concentrations were determined by LC-MS/MS (rotenone and dinoseb), GC-MS/MS (endosulfan) or ICP-MS (mancozeb).

Our results show that the 96-hour exposure was most adverse for all pesticides. Interestingly, internal concentration measurements showed no accumulation of the tested pesticides. Aside from the 96-hour exposure, the most adverse exposure scenario was pesticide dependent. For Dinoseb, mancozeb, and rotenone, exposure scenarios with most severe effects on mitochondrial dysfunction were 24 hours without recovery, 24 hours with 72 hours recovery, and 24 hours divided over 4 days, respectively. Remarkably, the outcome was independent of the exposure scenario for Endosulfan.

These data indicate that safe exposure levels determined with 24 hours acute toxicity tests as default may not be predictive for long-term effects. As such, these tests may not be informative for risk and safety assessment of pesticides concerning neurodegeneration. Moving towards a novel regulatory framework, it is essential that real-life exposures and continued assessment of effects after exposure are taken into account. This could be accomplished by incorporating dedicated *in vitro* test paradigms, as well as modelling approaches.

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Evidence for negative renal health effects related to PFAS exposure

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The CHIASMA project aims to optimize a comprehensive set of New Approach Methodologies (NAMs) and integrate them into a robust framework to evaluate chemical and material safety without relying heavily on animal testing. These NAMs will be tested on chemicals from the PFAS family due to their health impacts, environmental persistence, and industrial applications. PFAS, known for their resistance to water, stains, heat, and biodegradation, have been linked to many health issues such as hepatotoxicity, reduced infant birth weight, disrupted lipid metabolism, and compromised immune responses. This research aims to investigate the impact of PFAS compounds on kidney cells using *in vitro* cell models (human RPTEC/TERT1 proximal tubule cells) and to explore whether environmental pollutants like PFAS adversely affect the function of renal cells. This can be studied by exposing cells to a range of concentrations of selected PFAS compounds. Viability assays, cell tracking experiments, immunofluorescence analysis, barrier function tests, and mitochondrial activity assays are being utilized to evaluate cellular responses. These methodologies provide a non-animal approach to investigating PFAS toxicity at a cellular level in cells relevant to human health. Preliminary results indicate that some specific members of the family, including PFOS, induce dose-dependent effects at relevant concentrations reflecting human health concern. Ongoing work is exploring mechanistic underpinning of these observations and expanding the chemical test group.

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The integration of *in vitro* cell-based and computational approaches to improve the toxicological hazard assessment of nephrotoxics

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The kidney is a main site of clearance in the body and therefore a common site for toxicity induced by chemicals. Human nephrotoxicity is poorly predicted using standard animal test methods. This is likely due to interspecies and intraspecies variations in the expression and functionality of xenobiotic transporters in the proximal tubule of the kidney. These transporters facilitate accumulation of toxic levels of chemicals in the proximal tubule. New approach methodologies (NAMs), including *in vitro* toxicity assays with human proximal tubule cell lines and physiologically based kinetic (PBK) modelling, have the potential to improve our understanding of the hazards and risks of nephrotoxics in humans. PBK-models can be used to translate *in vitro* effect concentrations to human equivalent nephrotoxic doses, an approach known as quantitative *in vitro-in vivo* extrapolation (QIVIVE).

In this study, new PBK models that include a multi-compartment kidney have been developed and evaluated for nephrotoxic substrates of the organic cation transporters and organic anion transporters (OCT and OAT, respectively). When including OAT and OCT-mediated active secretion of chemicals from blood into proximal tubule cells into the PBK models, the clearance from the blood of OCT substrate cisplatin and OAT substrates tenofovir and polymyxin B is increased and the chemicals accumulate in the proximal tubule cells. *In vitro* cytotoxicity and renal basolateral transporter kinetic data were generated using wildtype and OCT2 and OAT1 transfected renal proximal tubule epithelial cells (RPTEC/TERT1) and conditionally immortalized proximal tubule epithelial cells (ciPTEC) parent cell lines to parameterize the PBK models to predict proximal tubule concentrations and assess the contribution of the xenobiotic transporters driving nephrotoxicity.

Dose levels of cisplatin that cause nephrotoxicity in humans are simulated and compared to the incidence of renal injury in chemotherapy patients. In the PBK model for cisplatin, inclusion of active secretion resulted in plasma concentration-time profiles that fall within a 2-fold range of the *in vivo* measured plasma concentrations. *In vitro* concentration-response data for cisplatin in renal cell lines collected from literature showed a 600-fold variance in EC50 values. The concentration-response curves differed between assay type, cell type, serum percentages in exposure medium, and exposure time. This suggests that concentration-response relationships using internal concentrations in proximal tubule cells *in vitro* should be used for accurate QIVIVE. Moreover, when performing QIVIVE relating PBK model estimates of maximum proximal tubule concentrations *in vivo* to *in vitro* cell-associated effect concentrations, nephrotoxic doses in humans were better predicted than when PBK model estimates of maximum plasma concentrations were related to *in vitro* nominal effect concentrations, which is conventionally done in QIVIVE studies. This case study approach to predict safe dose levels with known nephrotoxics forms the foundation for a NAM-based hazard and risk assessment of lesser known nephrotoxics that are substrates for the same transporters in the proximal tubule.

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A breath of fresh air: Optimizing cell cultures with hemocyanin

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Oxygen is essential for mitochondrial function, ATP synthesis, and oxygen-sensitive enzyme activity. However, there is a misconception that culturing cells in a 21% saturated oxygen environment provides too much oxygen to cultured cells. In fact, due to the poor dissolution of oxygen in water evidence suggests most cells are oxygen deprived. Hemocyanins are copper-containing oxygen carriers in many molluscs and some arthropods and are thought to have evolved to specialize in low oxygen environments. Hence, we tested the ability of hemocyanin to increase oxygen distribution and delivery in static culture of the renal cell line RPTEC/TERT1.

RPTEC/TERT1 cells were seeded in Seahorse 96-well plates. After attachment, cells received hemocyanin-supplemented media (100 μ L medium per well), with daily imaging and bi-daily feeding. Digital phase contrast imaging confirmed a direct correlation between hemocyanin treatment and enhanced growth. By day 7, treated cells displayed increased proliferation, reaching confluency and forming maturation-associated domes by days 12-14. The experiment was repeated in 384-well plates (30 μ L medium per well), where oxygen limitations were more severe due to the small surface area (0.06 cm²). The cells struggled to survive in control conditions but thrived when treated with hemocyanin. This was the first successful RPTEC/TERT1 culture in 384-well plates in our lab, allowing full maturation for experimental use. These findings demonstrate hemocyanin's potential to improve oxygenation in cell culture, supporting cell growth, maturation, and differentiation into specialized cell types, such as proximal tubular-like cells.

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Publications on the neurogenic theory of depression: Is report quality improved by guidelines?

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Systematic reviews are approaches that move towards the principles of the 3Rs, by "reducing, replacing and refining" the use of animals in research. Systematic reviews use well-defined methods to iden-

tify, evaluate, and interpret available evidence in the literature with greater statistical power than individual studies. In addition to rigor in methodology, the validity of the evidence in systematic reviews also depends on the quality of the primary studies that are included in it. Incomplete reporting in primary studies makes it difficult to assess their validity. Guides for experimentation and reporting in animal research, such as the ARRIVE guidelines, should ensure that studies are conducted with methodological rigor and reported in sufficient details to be reproducible or appraisable. Assessing the quality of studies included in a systematic review is an important component, as biases (or systematic errors) exacerbate or underestimate the true effect of an intervention. Present study investigated the influence of guidelines for animal studies on the quality of publications included in a systematic review on the neurogenic theory of depression (protocol available at <https://osf.io/gmsvd>). A sample of articles (n = 23), selected within 267 articles published from January 31, 2020 to March 10, 2023 and retrieved from databases (such as Medline, Embase, Scopus and Web of Science), were assessed for internal and external validities using the Syrcle's Risk of Bias (RoB) tool and the adapted CAMARADES checklist, respectively. Data were manually extracted from full texts by one independent reviewer and analyzed using the Robvis package on the R software. Nearly 75% of the publications reported the use of experimentation guides for animal research, including one reporting to use the ARRIVE guidelines. According to the results of RoB tool, no publication was in high risk of bias. In 100% of publications, the risk of bias was unclear for most of items ("base line characteristics", "hidden allocation", "incomplete results", "report of selective result", and "other types of bias"). Studies reporting the use of any guide or no guide at all had between 25% and 75% of publications in low risk of selection bias (75% "animals were randomly allocated") or detection bias (50% "randomly selected for outcome", 25% "researchers were blinded"). The risk of selection bias assessed by the item "random allocation sequence" was low on 25% of the publications using guides (n = 17) contrasting to 0% in those studies using no guidelines (n = 6). According to CAMARADES checklist results, all publications regardless of whether they used guides or not, were in high risk of bias due to the lack of information about "conflicts of interest", "animal welfare", and "phenotype". These results indicate that recent literature reporting animal studies in the field of neurogenic theory of depression are on unclear risk of bias. There was a modest increase in the internal and external validities assessments when articles reported the use of guidelines for good experimentation or reporting practices in animal studies. In conclusion, unclear risk of bias in most assessments suggests that essential information as defined by the ARRIVE guidelines are still missing in many publications on the neurogenic theory of depression studies. Present conclusion can be more robust by extending the articles publication date to the year of ARRIVE publication (2010).

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In vitro strategy to determine the enzymatic exfoliating effect of cosmetic bioproducts

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Biological and environmental factors contribute to hyperkeratinization, causing rough and dry skin, a condition that can be mitigated through exfoliation. Exfoliation provides several benefits to the skin, including preventing clogged pores, reducing blackheads and pimples, and enhancing the absorption of treatment creams. While acid exfoliants promote skin renewal, they can cause redness, irritancy, and increase sensitivity. Enzymatic bioproducts exfoliants offer gentle skin exfoliation with minimal side effects. This study proposed a simple and functional *in vitro* strategy to determine the efficacy of cosmetic compounds in enzymatic exfoliation. The keratolytic effect of an active bioproduct with keratolytic properties was evaluated using *in vitro* skin models, including 2D and 3D (reconstructed epidermis) cell cultures. To ensure that the keratolytic effect was not associated with skin or eye irritation, tests were conducted following OECD Guidelines 439 (2021) and 491 (2023). To assess the enzymatic cell dissociation effect, 2D keratinocytes culture (HaCaT) were exposed to the bioproduct for 30 minutes, 1 hour, and 2 hours, and non-detached cells were stained with neutral red. Untreated cells and cells exposed to trypsin were used as controls. The keratolytic effect of the bioproduct at 2% and 5% concentrations was evaluated in a 3D cell culture model of reconstructed human epidermis (SkinEthic™ RhE) after 18 hours of exposure. A saline solution (0.9% NaCl) was used as a control. The 3D skin model was divided for histological analysis and gene expression analysis of filaggrin (FLG) and involucrin (IVL). The active bioproduct reduced cell adhesion to the plastic plate at a concentration of 10% across all time points (30 minutes, 1 hour, and 2 hours) and at 5% after 2 hours. Similar effects were observed with positive control trypsin, an enzyme with known keratolytic properties. Histological analysis showed greater detachment of the outermost layers of the stratum corneum at both 2% and 5% concentrations after 18 hours of active bioproduct exposure, confirming its exfoliating effect. Additionally, the active bioproduct stimulated FLG and IVL gene expression at 5% and 2% concentrations, respectively, contributing to skin barrier function and tissue renewal. No skin or eye irritation was observed following exposure to the active bioproduct. Both the 2D and 3D cell culture methodologies proved to be useful for evaluating the enzymatic exfoliating effect of the cosmetic active bioproduct, by assessing effectively the keratolytic properties at different concentrations and exposure times, offering a reliable approach while reducing the dependence on animal models.

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In vitro biomarkers for predicting the efficacy of cosmetic hair growth stimulants

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Wound-induced hair neogenesis (WIHN) is a complex process that's involves progenesis, morphogenesis, immunomodulation, and neovascularization, and it provides a model for understanding mammalian hair follicle formation. Double-stranded RNA (dsRNA) released from damaged cells triggers Toll-like receptor 3 (TLR3) activation, leading to the upregulation of hair follicle biomarkers (stem cell markers and morphogenic factors) essential for hair follicle formation and maintenance. Due to the biological complexity of hair regeneration, understanding its mechanisms has become a key focus in the treatment of hair loss, a common aesthetic concern that has driven the development of various cosmetic treatments aimed at promoting hair growth. This study aimed to propose a simple, functional, and *in vitro* approach to predict the efficacy of cosmetic compounds in promoting hair growth. To achieve this, the expression of genes associated with hair follicle progenesis and hair morphogenesis was evaluated over time, identifying the cyclic gene induction that leads to hair neogenesis. Primary human keratinocytes HEKn (Thermo Fisher Scientific) were exposed to Poly (I:C) (20 µg/mL), a synthetic analog of dsRNA that binds to TLR3 and activates the canonical hair development pathway. Untreated cells were analyzed as negative control. HEKn cells were exposed to Poly (I:C) for three different time periods (24, 48, and 72 hours), and gene expression was analyzed using RT-PCR. RNA was extracted using TRIzol, qualified and quantified with a Nanodrop One, and converted into cDNA using a thermal cycler (BIO-RAD). Gene expression was measured with a QuantStudio 7 Pro (Thermo Fisher Scientific) and Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) gene was used as housekeeping gene. Within the first 24 hours, CD200, KRT15, and GLI1 gene expression increased in HEKn cells exposed to Poly (I:C), followed by a rise in LGR5 and SHH gene expression after 48 hours. During the initial phase of keratinocyte differentiation into a proto-hair follicle, CD200 prevents immune self-destruction by inhibiting lymphoid cell detection. KRT15 and GLI1 confirm stem cell progression and hair follicle progenesis. The potentiation of Wnt canonical signaling in the hair follicle is maintained by LGR5, while the morphologic transition into the hair anagen phase is driven by SHH after 48 hours. Seventy-two hours after the 24-hour Poly (I:C) exposure, epidermal cells adjacent to the follicular area invaginate towards the dermis, mediated by LEF1 induction, preceding the formation of follicular condensates that establish the hair root. These results demonstrate that analyzing the expression of selected genes at specific time points effectively predicts the biological efficacy of treatments targeting hair follicle development. This approach presents a valuable tool for investigating the molecular mechanisms involved in hair follicle formation and regeneration.

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Sexually dimorphic responses of flies to chronic variable stress

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Model organisms are non-human species, which allows for a mechanistic understanding of human diseases and treatments. Most organisms used in neuropsychopharmacology are vertebrates. However, their use has been criticized due to the ethics and animal welfare aspects. Besides, using vertebrates presents disadvantages regarding experimental time, financial costs, and the necessary environment to raise them compared to tiny organisms, such as flies or nematodes. Initiatives to decrease the number of vertebrate animals in research have applied the principles of reduction, refinement, and replacement, known as the 3Rs. Invertebrate animals, such as the fruit fly *Drosophila melanogaster*, may replace the vertebrates. Flies possess some aspects of neurobiology homologous to vertebrates, such as serotonin (5-HT) and dopamine neurotransmissions. As laboratory animals, flies have contributed to the understanding of genetics for over a century and, more recently, have been helpful in investigating the mechanistic basis of neuropsychopharmacology. They present some advantages compared to vertebrates including easiness of breeding, low raising costs, and fully described genome and proteomes. Previous studies suggest that male flies react to stressors, such as cold, heat, starvation, or their combination, by reducing sucrose preference (SP) and 5-HT amount, as observed in vertebrates. The present study aimed to evaluate how sexual dimorphism affects the responses of flies to chronic variable stress (CVS). Male and female flies were randomly allocated to the following groups (n = 63 to 76/group): negative control (unstressed, fed), positive control (unstressed, food-deprived ~20 h before the behavioral test), and stressed (3-day-CVS, including cold, food deprivation, heat, cycle inversion and an additional ~20 h of food deprivation before the behavioral test). The behavioral test was performed in the lane-maze apparatus on the fourth day. 5-HT-positive cells in the brains of non-tested flies (n = 6 to 17/ group) were detected by immunohistochemistry. Males had higher locomotion (6.98 [95% CI 6.55-7.44]) than females (6.34 [95% CI 5.97-6.72]), independent of the condition. Independent of sex, CVS flies presented higher SP (male: 0.59 [95% CI 0.52-0.65], female: 0.47 [95% CI 0.39-0.54]) than food-deprived (male: 0.36 [95% CI 0.28-0.44], female: 0.20 [95% CI 0.12-0.29], p < 0.0001), or unstressed ones (male: 0.20 [95% CI 0.10-0.29], female: 0.03 [95% CI -0.06-0.11], p < 0.0001), and food-deprived flies presented higher SP than unstressed (p = 0.0056). The immunohistochemistry showed that the number of 5-HT-positive cells was larger in CVS females (742004.4 [95% CI 550255.6-1000572.0]) than in unstressed females (443412.6 [95% CI 312741.5-628681.4]) and CVS males (1042189.0 [95% CI 755916.9-1436876.0]). In conclusion, sexual dimorphism affects the responses of flies to CVS. Male behavior was slightly more susceptible to stress than females, while 5-HT transmission in females seemed more affected by CVS than males. This study indicates that the sex of flies is a relevant independent variable and should be considered in further studies contributing to the advance of the use of flies in this field.

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ADME processes of thiabendazole in human and rat *in vitro* models

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Introduction: Thiabendazole (TBZ) is a food preservative, anti-fungal agent and anthelmintic drug which prevents the polymerization of beta-tubulins. Its major metabolite 5-hydroxy-thiabendazole (5-OH-TBZ) has been found in the urine of over 90% of the Swedish population between 2000-2017. Traditionally human health risk assessment is conducted *in vivo*, but there are indications that TBZ is only hepatotoxic in rats while being hepato- and nephrotoxic in mice and humans. In human liver microsomes it has been shown that the major metabolite 5-OH-TBZ can undergo metabolic activation to form nephrotoxic molecules. Organic cation transporter (OCT) is believed to be involved in the uptake in the proximal tubule cells. TBZ is first metabolized by CYP1A2 in the liver and then further metabolized by UGT, SULT and GST. AIMS. We aim to investigate interspecies differences in the absorption, distribution, metabolism and excretion (ADME) of TBZ in liver, kidney and gut microbiome *in vitro* systems using a tiered approach.

Materials and methods: *In vitro* models including human and rat renal subcellular fractions, RPTEC/TERT1 cells, human and rat primary hepatocytes and human gut microbiome samples were exposed to TBZ or 5-OH-TBZ at different timepoints. The formation of different metabolites over time was explored using LC-MS. Additionally the renal transporter involved in the uptake of TBZ will be studied using suitable OCT inhibitors and quantified by transported TBZ.

Results and discussion: Exposure of 99 µM TBZ induced thioredoxin reductase 1 (TXNRD1) in RPTEC/TERT1 after 24 h exposure. Kinetic experiments in RPTEC/TERT1 and primary hepatocytes were performed with non-toxic maximum concentrations of 10 µM TBZ. Phase I metabolite 5-OH-TBZ was detected in rat liver microsomes. Phase II 5-OH-glucuronide formation was detected in human and rat renal microsomes. There was no 5-OH-sulfate measured in human and rat renal cytosol. Additionally, TBZ does not seem to be metabolized by the human gut microbiome.

Conclusion: Phase I and II metabolites of TBZ were detected in human and rat liver and kidney *in vitro* models. The experimental data will be integrated into physiologically based kinetic (PBK) models. This will help to improve these *in silico* models and lead to more accurate xenobiotic human health risk assessment.

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Evaluating markers of oxidative stress and inflammation in RHE models exposed to combined UV radiation and pollution

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The use of alternative methods to animal testing, such as Reconstructed Human Epidermis (RHE), represents a significant advancement in the assessment of cosmetic safety and efficacy. This *in vitro* model provides a more accurate representation of human skin responses, providing reliable alternative to animal testing while improving studies' predictability and ethical responsibility. Its application supports the increasingly strict regulatory requirements and science's commitment to relevant, robust, sustainable and innovative practices. UV radiation and environmental pollution are key contributors to oxidative stress and reactive oxygen species (ROS) production, particularly when combined, thus RHE models can be a valuable tool for evaluating not only their harmful effects but also the protective effects of UV filters and antioxidants on the skin. Therefore, this study aimed at evaluating the oxidative stress biomarkers induced by the exposure to UV radiation combined or not with pollution, as well as at assessing the protective effects of 1% α -tocopherol, used as a positive control. The RHE models were exposed to UVA radiation (6 J/cm²) from a solar simulator and nebulization of particulate matter (PM 2.5) using the Vitrocell Cloud™ equipment. Culture media was collected to ELISA assay after 1 h and 24 h of treatment for the evaluation of pro-inflammatory mediators' production, while the RHE models were collected and frozen at 4 and 24 h for subsequent aryl hydrocarbon receptor (AHR), glutathione peroxidase (GPX), superoxide dismutase (SOD) and IL-18 gene expression evaluation via RT-qPCR. The exposure of RHE models to UV radiation and pollution induced a substantial increase in the release of the pro-inflammatory mediators TNF- α , IL-10 and IL-18, mainly 24 h after exposure of both UV radiation and pollution. Moreover, IL-6 showed a slight increase, also mainly 24 h after exposure to both environmental stressors. Additionally, vitamin E 1% reduced the release of TNF- α , IL-6 and IL-18, which are involved in acute inflammation, as well as in the propagation of the skin damage in many inflammatory cutaneous conditions. IL-18 is also involved in photosensitizing responses to xenobiotics. Vitamin E 1% also reduced the expression of SOD, GPX and IL-18 after 24 h of UV and pollution exposure. In conclusion, the RHE model effectively demonstrated its capacity as a predictive model that can be used to evaluate the oxidative stress and inflammation induced by UV radiation and pollution. The antioxidant 1% α -tocopherol reduced these effects, highlighting its protective role. These findings reinforce the value of *in vitro* models and standardizations for the studying of environmental damage and testing new protective agents in cosmetic research.

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Towards animal product-free culture: HaCaT adaptation to a chemically defined and FBS-free system for enhanced biological relevance

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Replacing animals for new approach methodologies (NAMs) is an ethical and regulatory priority. However, many *in vitro* methods rely on fetal bovine serum (FBS), which introduces variability, contamination risks, and ethical concerns. Its replacement is essential for the development of standardized human-based models. This study investigated the adaptation of human adherent cell lines to chemically defined media, animal-product-free, aligned with Good Cell and Tissue Culture Practice (GCCP 2.0) guidelines. HaCaT cells (a human keratinocyte cell line) were used as a model to assess the adaptation to a chemically defined and FBS-free system. Except for FBS, the cell culture maintenance was whole animal-product-free. A gradual adaptation process was implemented by progressively reducing the FBS and supplementing the medium with recombinant human growth factors and glucose. After adaptation, HaCaT serum-free (HaCaT-sf) cells were seeded on E-plates at 5.0×10^3 cells/well (HF(A)) and 7.0×10^3 cells/well (HF(B)) in Epilife Medium supplemented with S7. HaCaT cells cultured in DMEM supplemented with 10% FBS were used as the control (HT). Cell proliferation and adhesion were monitored in real time using the xCELLigence RTCA Single Plate (SP) system. This study showed that adaptation process effectively supports cell viability and proliferation. Medium renewal at 46 h positively affected the proliferation of the adapted cells, with an up in the slope of the growth curve. Epilife + S7 with 4.5 g/L glucose sustained HaCaT-sf growth at a gradual pace, and initial cell density influenced late-stage proliferation, as HF(B)'s CI outperformed HT(A)'s CI during 72 h. This positive response to nutrient renewal highlights its importance in serum-free *in vitro* protocols. The adapted systems demonstrated a performance equivalent to or superior to that of conventional media, thus enhancing biological relevance. In addition to replacing animal products and including more accessible cell lines. Future cytotoxicity assays with HaCaT-sf cells will explore their potential applications in human-relevant toxicological testing.

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Advancing chemical safety assessment with a human-based cytotoxicity model

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The replacement of animal-derived materials in toxicological testing is important for ethical, scientific, and regulatory advancements. However, many *in vitro* methods still rely on non-human cells (e.g., Balb/c 3T3) and animal-origin products, limiting the full transition to human-based and animal-free testing strategies. This study investigates the feasibility of normal human gingival fibroblasts (hGF) as an alternative model for the acute cytotoxicity assay recommended by OECD Guideline (GD) 129. To assess the suitability of hGF as a human-based model, we conducted Neutral Red Uptake (NRU) assays to evaluate cell viability and dose-response effects of control substances, such as sodium dodecyl sulfate (SDS), with IC₅₀ determination. The NRU assay with hGF resulted in an IC₅₀ of 43.52 µg/mL for SDS, closely matching expected values from murine cell-based protocols, reinforcing the reliability of human cells for cytotoxicity evaluation. Furthermore, viability assessments in low-serum conditions (< 5% FBS) showed that 2.5% FBS provided the best response after 24 hours, while media containing 0.63%, 0.31%, and 0.16% FBS exhibited equal or superior absorbance values compared to 5% FBS. Except for traditional medium FBS-containing, all reagents used in adapted hGF cultures were chemically defined and animal-product-free, including basal media, the dissociation solution TrypLE™ Express, and the cryopreservation solution Synth-a-Freeze™. To further transition toward animal-free testing, future studies will implement hGF in a fully serum-free system to evaluate its cytotoxicity response, focusing on cell adaptation to chemically defined, animal-product-free media. By replacing murine cells and minimizing animal-derived supplements, this approach contributes to the development of scientifically robust, cost-effective, and human-relevant chemical safety tests. These findings align with global regulatory efforts to modernize *in vitro* toxicology and promote the adoption of non-animal materials in cell and tissue culture, reducing invisible animal suffering in scientific research.

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Measuring uncertainty of acute cytotoxicity results using the methodology of OECD Guidance Document 129

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Despite the use of strict acceptance criteria in validated methods for *in vitro* toxicology safety non-animal methods, the evaluation of statistical uncertainties in the results and their impact on the reproducibility and reliability of these methods is limited. Technical and operational variations in intra- and inter-laboratory settings can interfere with the results, and measurement uncertainty estimations are crucial for a better understanding of the potential sources of errors and ensuring that the assays are under control. The measurement uncertainty characterizes the dispersion of values, which can be attributed to the measurement results. This study applied the principle of propagation of distributions using Monte Carlo Simulation (MCS) to calculate the measurement uncertainty for the half-maximal inhibitory concentration (IC₅₀) of sodium dodecyl sulfate (SDS) using OECD Guidance Document 129. We focused on acute oral cytotoxicity *in vitro* and developed a specific tool for this purpose. The MCS method enabled the identification of the contributions to the measurement uncertainty of IC₅₀ and their confidence intervals for the *in vitro* cytotoxicity assay recommended by OECD GD 129. The Hierarchical Bayes (Laplace) method was applied to combine the results from multiple sources, ensuring robust and statistically sound estimation. In conclusion, this study demonstrates the effectiveness of the Monte Carlo statistical model in quantifying the measurement uncertainty of sodium dodecyl sulfate (SDS) IC₅₀ values, with a comprehensive analysis yielding a combined IC₅₀ of 34.5 µg/mL with a combined uncertainty of 1.21 µg/mL. These findings highlight the importance of incorporating metrological evaluations to increase the reliability of *in vitro* methods. By enabling more informed decision-making, this study can significantly contribute to the advancement of toxicological research.

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Systematic reviews as a tool for enhancing validity in animal stroke research

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Ischemic stroke is a leading cause of global disability, yet its pathogenic mechanisms remain poorly understood, limiting the effectiveness of current treatments. This issue is compounded by animal models that inadequately reflect relevant patient characteristics, such as health status and age, and the predominant use of male subjects, despite stroke affecting all genders. Consequently, treatments validated in these models may prove ineffective or even harmful for most patients. Preclinical systematic reviews offer a robust method to identify knowledge gaps and inform future animal experiments while aligning with the 3Rs principle. They support replacement by generating new insights from existing data without the need for additional animal use, promote reduction by ensuring that animal experiments are conducted only when fully justified, and enhance refinement by improving study design and animal welfare conditions. Understanding how sex, age, and comorbidities influence stroke outcomes is essential for improving the clinical relevance of animal studies while also advancing the 3Rs, leading to more ethical and effective preclinical research.

To address this, we conducted three systematic reviews examining the impact of sex, age, and common stroke-related comorbidities (diabetes, hypertension, obesity) on stroke outcomes and treatment efficacy. Following preregistered protocols, we searched four databases and performed eligibility screening, data extraction, and risk of bias assessment in duplicate. We included over 600 publications, computed normalized mean difference effect sizes, and applied random effects meta-analysis to combine effects. Our findings show that females exhibit 34% smaller infarcts than males, though this advantage disappears in aged and ovariectomized animals. Advanced age and comorbidities were associated with worse outcomes – obese animals exhibited 40% larger infarct volumes, while hypertensive animals showed a > 200% increase compared to normotensive controls. Meta-regression analyses further revealed age- and comorbidity-specific variations in treatment responses.

These findings provide valuable insights into key determinants of ischemic stroke modeling and highlight factors contributing to the poor translation of preclinical findings. Our study identifies sources of variability that refine animal models, enhancing their clinical applicability and improving the predictive value of preclinical stroke research. This ultimately contributes to the development of more effective and personalized therapeutic interventions in clinical settings.

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A novel nose-to-brain drug delivery prediction model utilizing an *in vitro*-*ex vivo* combination

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Effective delivery of drugs to the brain remains a challenge following systemic treatment, due to the blood-brain barrier (BBB). The purpose of the BBB is to protect the brain against damage by potentially toxic chemicals in the systemic circulation. However, this results in preventing approximately 98% of small molecules in the systemic circulation to reach the brain. This has made effective treatment of neurological disorders a challenge. An alternative route of drug administration has increasingly been investigated, namely the intranasal route, since it allows bypassing the BBB through direct nose-to-brain drug delivery.

Although animal models are typically utilized during drug development for testing prior to clinical trials, an increasing need for alternative models has been identified for higher physiological relevance, cost-effectiveness, high throughput and more ethical research. *In vitro* cell culture-based models provide one such alternative, but traditional flat cultures are physiologically less relevant. To address these shortcomings and improve the correlation between *in vitro* models and the *in vivo* environment, more complex three-dimensional (3D) cell models such as spheroids have been developed. *Ex vivo* animal tissue models have been used to predict drug permeation across the nasal epithelium and these tissues are usually sourced from discarded waste byproducts of meat production processes. The use of excised tissue in this way reduces the need for breeding animals specifically for research purposes.

In this study, we developed a novel combination model consisting of *ex vivo* sheep nasal epithelial tissue and an *in vitro* 3D U-87MG human glioma spheroid model to enable investigation of both nasal drug delivery across the olfactory epithelium and drug uptake into the brain. The aim of this study was to develop and characterize the *in vitro*-*ex vivo* combination model using 3D U-87MG human glioma spheroids, grown in a clinostat-based rotating bioreactor, combined with excised sheep nasal epithelial tissue in a Sweetana-Grass diffusion apparatus. To qualify the newly developed model for nose-to-brain drug delivery studies, membrane permeation and drug uptake within the brain spheroids was investigated using two model compounds with different membrane permeability properties (i.e., caffeine and acyclovir).

Studies were conducted in six-fold, with the membrane permeation of the model compound solutions determined in the absorptive direction, i.e., apical to basolateral. The U-87MG human glioma spheroids were placed in cell culture medium in the basolateral chamber, and every 30 min for 3 h, 200 µL samples as well as one spheroid were removed from the basolateral chamber and analyzed for model compound concentration using high performance-liquid chromatography linked to mass spectroscopy. The percentage drug transport was calculated from the drug content within the growth medium sample, and drug uptake was determined from the spheroid drug content.

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Assessment of cytotoxicity and mechanistic insights from a combination of three independent anatomical regions within the respiratory tract using *in vitro* 3D human models following exposure to a 1R6F reference cigarette

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New Approach Methodologies (NAMS) are being implemented for inhalation toxicity to deliver improved safety assessment and hazard identification. There is an increased focus to investigate products in more physiologically relevant environment. *In vitro* human 3D models are a relevant method for analysis of toxicity and modelling downstream effects such as inflammation and additionally complying with the principles of 3Rs. This study utilized models from Epithelix Sarl (Switzerland) and included MucilAir™, SmallAir™ and AlveolAir™ in their response to whole smoke from a 1R6F Kentucky Reference Cigarettes. The exposure model covered the response from the upper airway, lower epithelium (bronchiolar region) and the alveolus epithelium all cultured at the Air Liquid Interface (ALI) covering all the main anatomical regions of the lung.

MucilAir, SmallAir and AlveolAir tissues were exposed using a Vitrocell® VC10® smoking robot to 1R6F Kentucky Reference cigarettes (smoked to ISO 20778). Whole Smoke was diluted at varying concentrations with flowing air. Liquid traps were placed at ALI and analyzed for nicotine, which was used as a marker for delivered dose.

In this study, we used the Lactate dehydrogenase (LDH) release, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt) to assess toxicity. Trans-epithelial electrical resistance (TEER), Cilia Beat Frequency, a pro-inflammatory cytokine panel and RNA analysis were performed to provide molecular insights. Post exposure tissues were left for 24 h recovery after treatment prior to analysis.

Exposure of MucilAir, SmallAir and AlveolAir to whole smoke resulted in different levels of viability and different mechanistic insights.

This study was a proof of concept suggesting that different anatomical locations of 3D human lung models can be used for safety assessment and for elucidating pathways of cytotoxicity through mechanistic insights. Difference in response between MucilAir, SmallAir and AlveolAir demonstrate that the tissue models should be chosen according to the physical characteristics of the aerosol, including particle size.

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Transpacific eye hazard journey: Reproducibility of the STE0.5 for surfactants

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A Defined Approach (DA) for eye hazard identification according to UN GHS has been developed for surfactants (DASF). The DASF is based on the combination of Reconstructed human Cornea-like Epithelium (RhCE) test methods (OECD TG 492) and a modification of the Short Time Exposure (STE) test method. The STE test method, an already OECD endorsed TG (TG 491, 2023), was optimized to discriminate between UN GHS Cat. 1 and not Cat. 1 surfactants (herein after referred to as STE0.5 for surfactants). The STE0.5 test method for surfactants is based on the same principle and techniques, except that a five-minute exposure to a concentration of 0.5% of a test chemical, and a different cut-off, is used to distinguish Cat. 1 (viability < 20%) from not Cat. 1 (viability ≥ 20%). Within- (WLR) and Between-laboratory reproducibility (BLR) of the OECD adopted STE test method was evaluated during validation studies. The purpose of the current study was to evaluate the WLR and BLR of the STE0.5 for surfactants to support its inclusion in OECD TG 491.

The WLR and BLR of the STE0.5 for surfactants were evaluated using a subset containing approximately two-thirds of the full surfactant set used to assess the performance of the DASF (N = 50). The subset includes the three UN GHS categories and the full range of viabilities. The surfactants were tested at a South American laboratory (*Laboratorio de Métodos Alternativos*, Argentina), and the results were compared with those obtained from Japan (data from the validation study, kindly provided by validation team members). Within each laboratory, each surfactant was tested in three independent runs, each containing three replicate wells. The WLR and BLR were above 90%, demonstrating excellent agreement within and between the laboratories.

These results indicate that the STE0.5 for surfactants, which is part of the DASF, is a reliable method for identifying UN GHS Cat. 1 surfactants in the first tier of the top-down approach or the second tier in a bottom-up approach. This represents a significant advancement, enabling eye irritation hazard assessments within a complete *in vitro* framework. Importantly, the excellent reproducibility supports the inclusion of the STE0.5 for surfactants in OECD TG 491. When applied as part of the DASF described in TG 467, it can be used for hazard classification and labeling of surfactants across the three UN GHS categories (Cat. 1, Cat. 2, and No Cat.).

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Increasing animal reuse: Novel approaches to achieving a 3Rs reduction of naïve animals in nonclinical safety assessment, benefitting supply chain sustainability, higher ethical standards and enhanced animal data reliability

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In Pharmaceutical Development, it is a regulatory requirement under ICH guidelines to perform nonclinical *in vivo* safety assessment studies prior to entry into Human (eH) clinical trials. Most *in vivo* nonclinical studies, including Regulatory Toxicology studies, typically default to using naïve animals; however, there is already a precedent set for reusing animals in specific study types, such as Safety Pharmacology and Pharmacokinetic studies. When implemented correctly, increased animal reuse offers an ethical opportunity to reduce significantly the number of naïve animals entering the supply chain. In addition, published literature, and the authors own experience, provides evidence to suggest that animal data reliability and potentially translatability, could be enhanced by reducing stress levels in animals through optimizing and/or increasing animal acclimatization and procedural training durations.

The discussion around increasing animal reuse within Regulatory Safety Assessment of Pharmaceuticals has been ongoing for most of the last decade, focusing most notably on non-human primates (NHP). From an ethical perspective, increased animal reuse can apply to all species (rodent and non-rodent), and can be achieved by strategic planning, cross industry collaborations and the establishment of new approaches such as multi-site *in vivo* phases, sharing reuse colonies and enabling non-terminal end-points.

Our poster/presentation provides a full discussion of these new approaches. The ethics surrounding animal reuse is unequivocal in terms of global legislation and guidelines on the subject. Implementation of reuse can only proceed when the health of the animal is assured, the lifetime burden does not exceed acceptable limits, and the animal is free from pain or distress. It is the authors experience and belief that the number of opportunities for compliant and ethical reuse exist in greater numbers than are currently achieved. With increased reuse the number of naïve animals bred to enter the supply chain will reduce and the animals within facilities will become better acclimated, more compliant to procedures and exhibit less stress related behaviors and physiological responses that can affect their biological parameters and subsequently the data interpretation.

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Science communication: Bridging the gap between animal research and new approach methodologies (NAMs): A knowledge sharing campaign

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The integration of NAMs into biomedical research is critical for advancing ethical practices in life sciences. However, a gap exists in understanding between animal researchers and the broader NAM community. This abstract presents a knowledge-sharing campaign designed to bridge this gap by introducing NAMs, simplifying their complex concepts through relatable analogies, and raising awareness of their benefits. The campaign aimed to increase interest in NAMs among animal researchers and promote the adoption of alternative methodologies.

Structured around five key content pillars – NAM Definition and Importance, Types, Benefits, Challenges and Solutions, and Success Stories – the campaign included three strategically timed social media posts per pillar. The campaign effectively leveraged LinkedIn as its primary platform, targeting key organizations such as the European Animal Research Association (EARA), the Federation of European Laboratory Animal Science Associations (FELASA), the European Federation of Animal Science (EAAP) and more. By tagging these organizations in posts, the campaign directly engaged their established communities, ensuring the content reached a relevant audience. These posts were shared on LinkedIn, leveraging peak user activity periods (14:00 CET, Wednesdays) to maximize reach and engagement. The content was grounded in the latest scientific literature, using analogies that made complex NAM concepts accessible and engaging for animal researchers.

The effectiveness of the campaign was assessed through a combination of mid- and post-campaign evaluations, including an interactive poll and a Q&A session. Key metrics indicated heightened engagement, with Altartox's LinkedIn page experiencing a 139-follower increase on days when campaign posts were shared. Posts averaged 667 impressions per post, and the click-through rate was 4.5%, reflecting strong visibility and audience interaction. A poll revealed that 100% of respondents correctly identified the key benefits of NAMs over traditional animal testing, highlighting the campaign's success in promoting understanding and retention.

This campaign demonstrates the potential of strategic social media outreach as a relevant tool to bridge gaps between different communities within the life sciences. It also illustrates how such campaigns can be leveraged to integrate NAMs into research curricula and training programs, promoting a culture of responsibility, ethics, and innovation. The findings underscore the importance of raising awareness and promoting the use of NAMs in research, aligning with the 3Rs (Replace, Reduce, Refine) principles and advancing the ethical evolution of scientific practice.

Communication campaigns can play a crucial role in promoting NAMs, providing insights on how to integrate these methodologies into scientific education. By utilizing modern communication tools, such as social media, and employing targeted educational strategies, we can foster a more responsible, informed, and ethically engaged research community.

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Understanding the range of phenotypic responses for the embryonic zebrafish developmental toxicity assay

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Embryonic zebrafish assays have been utilized to screen for chemically induced developmental toxicity. Many research groups employ different experimental protocols and often report toxicity as a combined mortality and altered phenotype metric. However, specific information on phenotypes is useful in chemical hazard assessments and can potentially inform on mechanisms of action. Here we investigate the incidence variability of phenotypes that can occur within and between laboratories across varying experimental protocol conditions. An interlaboratory study with three participating laboratories tested a suite of 42 blinded chemicals at five or more concentrations each using four combinations of experimental conditions: embryos with chorion intact or removed (dechorionated) and exposure to test chemical via a static or static-renewal protocol. Briefly, single zebrafish embryos (approximately 4-6 hours post fertilization) were placed into individual wells of a 96-well plate for a 5-day exposure to blinded chemicals, the positive control substance (3,4-dichloroaniline, DCA, 0.1-50 μ M, n = 1-2 embryos per concentration per plate), and the vehicle control (0.5% dimethyl sulfoxide, n = 11-12 embryos per plate). Phenotypes were first recorded using laboratory-specific phenotype recording terms. Due to variations in how these were reported, these laboratory-specific terms were annotated to Zebrafish Phenotype Ontology terms and further grouped into granular and general categories based on anatomical region to assist with cross-laboratory comparisons. Benchmark concentrations (BMC) per phenotype term and experimental conditions for each laboratory were calculated for all chemicals. The BMC values for DCA varied between 3-45 μ M across laboratories and experimental conditions. For DCA, the most specific altered general phenotype across all three laboratories was head defects, including abnormal head shape and snout/jaw defects. Some of these responses were dependent on the exposure scenario, with lower BMC values observed under static-renewal conditions for two laboratories. Ongoing analysis for the remainder of the 42 blinded chemicals will determine if head defects remain a sensitive indicator for developmental toxicity or if phenotype patterns are chemical- or laboratory-specific. An additional evaluation of vehicle-treated embryos also demonstrated variability across laboratories and experimental conditions. One laboratory reported higher incidence rates for altered phenotypes (0-16% affected) compared to the other two laboratories (0-3% affected), suggesting that experimental handling may have been a factor. For the laboratory with the higher incidence rates, some of the phenotypes observed at rates greater than 3% included craniofacial deformities and scoliosis. Overall, these results can help to understand the landscape of variability within and across laboratories that implement unique zebrafish testing protocols. While there were similarities

in responses for the selected positive and vehicle controls, the range of different phenotypes reported by each laboratory indicates that inclusion of refined phenotypes in assessments can enable sensitive findings.

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Web application to classify and subcategorize skin sensitizers using human data

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Current approaches that use human data to assign skin sensitizers to United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) potency subcategories overlook the number of sensitized test subjects, potentially leading to an underestimation of potency. To address this concern, human predictive patch tests (HPPT) data collected to support evaluation of non-animal approaches were used to develop a modified approach for assigning skin sensitization potency subcategorizations based on GHS. The modified classification approach improves upon previous methods by considering the number of sensitized human test subjects and addressing uncertainty in assay results. Additionally, the approach includes a weight-of-evidence method to consolidate multiple HPPT results into a single GHS classification for each chemical. However, the approach involves complex decision tree logic that may be challenging for most users. To allow a broad audience of users to apply this modified approach to their own HPPT data, we have developed an open-source R Shiny-based web application, HPPT App. The app provides an interactive platform that imports user-submitted HPPT data and applies the modified GHS classification approach to classify and subcategorize skin sensitizers. To classify a substance for skin sensitization hazard, the app first derives the two response values defined within the modified approach: DSA1+, which indicates the dose that sensitizes one test subject, and DSA05, which indicates the dose that sensitizes 5% of test subjects. The app then assigns classifications for each test result. Under standard GHS classification rules, a substance can be classified as a strong sensitizer (1A), a weak sensitizer (1B), or not classified (NC). The HPPT App enables a modified classification approach, addressing uncertainty by defining borderline ranges to assign ambiguous classifications in cases where the data show no evidence of strong sensitization (NC/1B) or provide no information for classifying skin sensitization (NC/1). It provides classifications for each substance based on three different classification modes. Depending



on the input data, the app may provide a binary classification (Category 1 [sensitizer] vs. NC [nonsensitizer]) or may be able to classify sensitizers as Category 1A or Category 1B sensitizers. Derived response values (DSA1+ and DSA05) and measures of classification reproducibility are also provided. The app output is provided as interactive tables that can be sorted, filtered, and downloaded. Additionally, the app website provides support documentation including information on the classification methodology, navigation guidance, and contact details. The HPPT app works with various types of data, such as test results from ingredients in consumer goods and cleaning products, highlighting its robust classification performance, applicability, and impact.

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Do we need best practice guidance in the design and reporting of *in vitro* research? A thematic analysis of user testing feedback using the RIVER recommendations

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In vitro models are widely used across the life sciences. They have direct implications for animal use as they provide evidence for future *in vivo* studies and present significant replacement opportunities. However, recent studies have demonstrated *in vitro* reporting is often inadequate and is a major contributor to irreproducibility.

To address this, we have developed a set of reporting standards – the RIVER (Reporting *In vitro* Experiments Responsibly) recommendations: a new, concise, reporting guideline specific to *in vitro* studies (doi:10.31222/osf.io/x6aut). The development of RIVER has been led by the NC3Rs in collaboration with an international Working Group

that includes researchers from academia and industry, statisticians, representatives from journals, funders, and regulatory organizations. To ensure that RIVER is fit for purpose, we conducted a user testing study to gather feedback from researchers. We then analyzed researchers' feedback and responses to identify common reporting issues by conducting a thematic analysis.

Eleven *in vitro* researchers who were in the process of writing a manuscript were recruited to user test RIVER. Participants were asked to use and implement RIVER when writing their manuscript. The completed manuscripts were reviewed by members of the research team and RIVER working group for alignment with the RIVER recommendations. An interview was then conducted to provide feedback to participants and to capture their views on RIVER. The transcripts from these interviews underwent thematic analysis to review the need for best practice guidance in the reporting of *in vitro* research and assess the need for additional guidance to support the design of experiments.

The sample was mainly from the UK with additional participants from Germany, Czechia and New Zealand, which included researchers across various career stages (PhD student – group leader) and utilizing a range of different *in vitro* models across various fields. While general methodological detail around reagents and equipment were well reported, a number of issues were identified, particularly around the correct identification of experimental and biological units and methods to address sources of bias.

Discussing missing information with participants highlighted a lack of understanding of several best practice concepts, particularly around when and how to implement measures to address bias in the *in vitro* setting. Where methodological details were not reported, the main reasons cited were that including these details is not standard practice or the participant not wanting to report processes that were not performed. Where further methodological detail was required, the participants were able to provide this verbally, indicating that information required for replication was either consciously or unconsciously omitted. Finally, a lack of guidance or examples in the literature of how to apply best practice concepts in the *in vitro* setting was highlighted as an issue and barrier to implementation.

In response to these findings, we are evolving RIVER into DRIVER (Designing and Reporting *In vitro* Experiments Responsibly), to address both the design and reporting of *in vitro* studies and incorporating in the feedback from participants. We will launch an online resource that will provide up to date guidance and practical advice on applying best practice across the research life cycle from the design through to the reporting of experiments.

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An *in vitro* test battery using human whole blood for immunotoxicity hazard identification: Proof of concept studies with dexamethasone and benzo(a)pyrene

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Immunotoxicity assessment is nearing a crossroads for approach and technology that is predicated on the mounting pressure for reduction and replacement of animals in toxicology research. The desire to replace animals has fueled an initiative to develop alternative approaches for hazard identification and safety assessment, collectively coined New Approach Methodologies (NAMs). The present work details the development and performance of a comprehensive NAM test battery using human whole blood for the assessment of immunotoxicity. The test system consists of a closed tube culture system containing whole blood diluted 1:3 in culture media with or without one of the following stimulants – monoclonal anti-CD3/CD28 antibodies, SARS-CoV-2 peptide pool, or influenza peptide pool. The known immunotoxicants dexamethasone (DEX; 0, 0.01, 0.1, and 1 micro-g/mL) and benzo(a)pyrene [B(a)P; 0, 0.025, 0.1, 0.4, 1.6 or 6.3 micro-M], used as model compounds, were added to the test system and incubated for 24 hours prior to immune assessments. Following culture, immune cells were identified and counted by flow cytometric immunophenotyping and assessed for natural killer (NK) cell activity and T cell activation. Supernatants were interrogated for proinflammatory cytokine concentrations. *In vitro* treatment with DEX resulted in suppression of cytokine production, NK activity, and T cell activation following stimulation with anti-CD3/CD28. In addition, cytokine production induced by viral peptide stimulation was significantly reduced by DEX treatment. Treatment with B(a)P resulted in suppression of cytokine production and nonsignificant reduction in T cell activation but did not impact NK activity. Immunosuppression by B(a)P was only observed following co-incubation of whole blood with an S9 fraction to allow metabolic activation of B(a)P and demonstrates that the system is amenable to testing compounds that require metabolism to produce immunotoxicity. These results provide evidence of immunotoxicity for the model compounds that reflect well established outcomes from *in vivo* studies. This human whole blood NAM battery shows strong potential for screening compounds for immunotoxicity hazard identification without relying on animal systems.

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Development and evaluation of an automated tool for assessing laboratory animal welfare

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Animal welfare assessments are essential for minimizing the likelihood of pain and distress in animals, while also increasing their potential to experience positive emotional states. In Canada, these assessments are now a requirement for regulatory compliance for all animals used in scientific research. However, standardized methods for evaluating laboratory animal welfare remain limited. Here we present the development and evaluation of an automated tool designed to assess laboratory animal welfare. The process began with a gap analysis to identify potential welfare indicators from animal data regularly collected in a centralized system across three Charles River facilities in Canada. We then conducted an online survey of veterinarians (n = 8), technicians (n = 38) and other laboratory animal professionals (n = 21) within these facilities to assess the severity of 174 animal-based welfare indicators. The results showed that veterinarians agreed on the severity of 165 indicators (70-100% agreement) and the severity of 80% of the indicators was consistently rated across each category of participants. The most consistently rated indicators (n = 139) were used to develop a severity-weighted scoring tool. To evaluate the scoring tool, the welfare scores of 628 mice were estimated and then compared to veterinary records and an *in vivo* welfare assessment. We found that 14% of mice had at least one mild welfare indicator recorded, and below 1% had severe to unacceptable welfare scores. These findings were consistent with veterinary records, mitigation measures (e.g., medical treatments, unscheduled euthanasia, etc.), and the *in vivo* welfare assessment, suggesting that the welfare assessment tool accurately reflects the welfare status of laboratory mice. In the following stages, we will evaluate the positive predictive value of the tool using historical data and will assess the tool validity for other laboratory animal species.

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Physiologically based pharmacokinetic (PBPK) modeling approach to quantify chemical distribution in brain and adipose compartments

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Physiologically based pharmacokinetic (PBPK) modeling can be applied to high-throughput applications to facilitate decision-making in drug discovery and risk assessment. However, high-throughput pharmacokinetic modeling is limited to those compartments included within the model, with brain and adipose compartments often lacking due to difficulties in calculating chemical distribution. For the brain, this is complicated by the presence of the blood-brain barrier (BBB), which limits chemical transport and partitioning into the brain. Adipose tissue is particularly complex, as it acts both as a storage compartment for lipophilic chemicals and a source of continuous internal exposure, as the chemical is subsequently released over time. Estimation of brain and adipose tissue concentrations are, however, important in assessments of neurotoxicity and long-term exposures to persistent chemicals. To address these challenges, we have added brain and adipose tissue compartments to the base PBPK model from the U.S. Environmental Protection Agency's *httk* R package (v2.2.2). The model workflow was executed in multiple stages including design of model structure, collection of model parameters, model development, curation of validation data, and model evaluation. For the brain compartment, the BBB permeability calculation utilized measured permeability values via transwell assays as well as predictions from a model built with collected measured results. The concentration-time profiles generated by the model overlapped for several tested scenarios including comparisons with experimental data and alternate PBPK model predictions in both brain and adipose compartments. As an example, the *C_{max}* values predicted by the model for brain and adipose tissues were within 2-fold of the experimental data measurements for both hydrophilic chemicals like acetaminophen (predicted *C_{max}* = 54.01, observed *C_{max}* = 74.03) and hydrophobic chemicals like benzo(a)pyrene (predicted *C_{max}* = 11.21, observed *C_{max}* = 9.59). Similarly, the brain *C_{max}* values predicted by the model fell within 10-fold of values predicted by the commercial GastroPlus PBPK model for 92 chemicals with potential developmental neurotoxicity. This alignment between the *httk*-based brain-adipose model's predictions and predictions from both commercial models and experimental data signifies the robustness of the model and its potential applicability for both drug development and chemical risk assessment.

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Spheroid bioassembly for a cartilage-bone interface model construction

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Osteochondral defects restoration represents a significant challenge for regenerative medicine, commonly evaluated using *in vivo* experimental tests. In tissue engineering, *in vitro* models that mimic complex natural tissues are being developed for regenerative therapies, minimizing animal suffering. In this context, spheroids derived from human adipose stem/stromal cells (ASCs) are used as a complex model to develop distinct microenvironments, such as bone and cartilage tissue. For the cartilage/bone interface bioassembly, a spheroid fusion must be well established, and this process is influenced by migratory cells capacity. The aim of this study is to evaluate the ability to bioassembly a cartilage/bone interface model fusing different spheroids for tissue engineering applications.

Fusion assay was performed using Bone-induced ASC/EA.hy926 spheroids, collected after 3 and 5 induction weeks (Bone); Chondro-induced ASC spheroids collected after 2 weeks (Chondro) and newly formed non-induced ASC spheroids (ASC NF). The spheroids were arranged in doublets: Bone/Bone, Bone/Chondro and Bone/ASC NF at 3 and 5 weeks of bone induction. The spheroid doublets were monitored by optical microscopy and their conjugate diameter (DC), contact diameter (DCT) and contact angle (AC) were measured for 7 days. The spheroid doublets were analyzed using confocal microscopy and histological assays at the end of the fusion process.

The Bone/Bone did not present complete fusion in weeks 3 and 5 (DC 821.3 and 843.3 µm, DCT 369.5 and 342.9 µm, and AC 139.4° and 136.83°, respectively). The Bone/Chondro did not present complete fusion in weeks 3 and 5 (DC 740.8 and 720.5 µm, DCT 366.5 and 342.2 µm and AC 143.6° and 145.7°, respectively). The Bone/ASC NF presented complete fusion in weeks 3 and 5 (DC 626.5 and 613.2 µm, DCT 433.5 and 397.8 µm and AC 175.7° and 167.3°, respectively). Confocal microscopy analysis demonstrated ASCs migration marked using CellTracker™ Green CMFDA (Invitrogen). ASCs cells culture moved from ASC NF spheroids to Bone spheroids during the fusion process. Hematoxylin-eosin histological analyses were used to confirm our results and demonstrated the Bone/ASC NF morphology after fusion. Histological images showed ASCs from ASC NF spheroid surrounding the Bone spheroid, indicating their potential influence on fusion kinetics.

In conclusion, it was observed that doublets with advanced maturation stages, such as Bone/Bone and Bone/Chondro at 3 and 5 weeks, do not achieve complete fusion after 7 days. In contrast, doublets involving undifferentiated spheroids, like Bone/ASCs at 3 and 5 weeks, achieve complete fusion, likely guided by ASCs' migratory capacity. We intend to create a macroscale model for regenerative medicine, developing a support hydrogel to facilitate spheroids migration and fusion, to produce macro tissue. This tissue engineering strategy could enable regenerative medicine therapies avoiding unnecessary invasive procedures in animal models.

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Navigating new approach methodologies: The RE-PLACE database as a Belgian resource for scientists

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Driven by scientific, economic, and ethical concerns, the relevance of the 3Rs Principle (Replacement, Reduction, Refinement) has gained significant importance over the past decades. New Approach Methodologies (NAMs) encompassing *in chemico* assays, *in silico* models and *in vitro* methods like organ-on-chip, -omics and high-throughput testing strategies, offer significant benefits for advancing (regulatory) science. While NAMs are generally considered as methods contributing to the replacement of animal tests, the term is also sometimes used in contexts referring to methods which use less animals compared to the original set-up.

The use of NAMs provides many benefits: (i) they allow more testing on a larger scale, (ii) they often make use of human-based material or data increasing the value of the extrapolation to human health, and (iii) they offer better insights into underlying (toxicity) mechanisms in cellular and molecular pathways. Altogether, this led to a growing interest worldwide. Despite the significant potential of NAMs, their (regulatory) acceptance and implementation is lagging behind.

As the field of NAMs is continuously evolving, it can be challenging to find relevant, reliable and up-to-date information about their actual use. Advancing the use of NAMs by bridging knowledge gaps may benefit from a bottom-up approach such as the “RE-Place” project. This na-

tional project addresses this challenge by creating a central, open-access database, available via www.re-place.be, that maps all available expertise in Belgium. It provides an inventory of already available NAMs, and the names of experts and organizations developing and/or utilizing these NAMs in Belgium. Since all information on the RE-Place website is freely available, it enables and enforces connections and knowledge exchange between researchers from different scientific areas.

In March 2025, the RE-Place database comprises nearly 300 models in biomedical research, regulatory testing and education. These methods have been submitted by 192 experts from 34 different organizations including industry, academia, spin-offs and scientific institutes. Most of the methods are *in vitro* methods, probably due to the ongoing developments in stem cells technology and organ-on-chip. Most of the submitted methods are situated in basic and applied research and have undergone peer review. Only a small fraction has been officially validated by an external validation body like the Organisation for Economic Co-operation and Development or the European Reference Laboratory for alternatives to animal testing.

By collaborating, scientists in Belgium can significantly increase the visibility of their work as the RE-Place team actively promotes progress in the field of NAMs through various communication and dissemination efforts. For example, scientists are offered the possibility to present their work during scientific events (i.e., study days, symposia and educational webinars). In addition, their research is further promoted via multiple channels (i.e., RE-Place website, social media platforms and newsletters). This exchange of up-to-date information and valuable know-how greatly facilitates broader outreach within the scientific community and beyond. As such, RE-Place stimulates the development and use of applications of NAMs with the ultimate goal of accelerating their adoption on a broader scale and contributing to the replacement of animal testing in the long term.

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***In vitro* primary human alveolar epithelium/endothelium and fibroblast co-culture model: A tool for investigating IPF and treatment strategies**

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Idiopathic pulmonary fibrosis (IPF) is a complex and fatal interstitial lung disease. While the etiology of IPF remains unknown, it is characterized by recurrent injuries and the dysregulated release of fibrotic and inflammatory factors (e.g., TNF- α , IL-6, IL-1), leading to abnormal wound healing and progressive scarring of the alveoli. The current paradigm suggests that TGF- β 1 plays a crucial role in the initiation of fibrosis by inducing extracellular matrix stiffening through the fibroblast-to-myofibroblast transition (FMT). Additionally, TGF- β 1 activates the epithelial-to-mesenchymal transition (EMT).

The aim of this study is to develop an IPF model using exclusively human primary cells. We present the co-culture and application of AlveolAir™, a tight epithelium composed of type I and II pneumocytes, cultured at the air-liquid interface, along with endothelial cells and lung fibroblasts.

AlveolAir™ and fibroblasts seeded on collagen-coated wells were stimulated with TGF- β 1 (20 ng/mL) and TNF- α (50 ng/mL) in the basal culture medium (DMEM). AlveolAir™ received daily apical stimulation with 20 μ L of culture medium for 72 hours. Nintedanib and Pirfenidone, used as reference antifibrotics, were tested concomitantly with the stimulation in the basal culture medium. To monitor FMT, α -SMA was analyzed, while EMT was primarily assessed by measuring multiple cytokines.

The expression and integration of α -SMA in the stress fibers were observed after stimulation, quantified by immunofluorescence, resulting in a significant increase in mean intensity. Following stimulation, the co-culture exhibited a significant increase in the release of matrix metalloproteinases, MMP1 and MMP3, in the basal culture medium – biomarkers also observed in patients with IPF. Exposing the stimulated model to Nintedanib or Pirfenidone significantly reduced α -SMA expression and cytokine secretion.

The described IPF-induced model has the potential to advance pre-clinical antifibrotic screening and enhance the understanding of molecular mechanisms in IPF.

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To pool or not to pool cells: A retrospective analysis in reconstitution of human nasal epithelium

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In vitro assessment of inhalation toxicity is a fast-growing field. Until now, in inhalation risk assessment, toxicity testing for regulatory purposes is performed only *in vivo* in line with OECD test guidelines. Therefore *in vitro* alternatives of human origin are urgently needed.

Human airway epithelial cultures cultured at the air liquid interface (ALI) in transwell as became a gold standard through years. However, although reproducing *in vivo* physiological characteristics of the corresponding tissues, primary human cell-based respiratory epithelia suffer at least two shortcomings: (i) difficulty to differentiate properly with high cell passage number and (ii) bearing genetic information from a single donor may give rise to a donor-specific outcome.

To overcome these problems, emerging strategies appeared pooling cells from several donors in the reconstruction process of human airway cultures. The aim of the current project is to describe pros and cons of this approach.

Epithelia (MucilAir™-Nasal-Pool) were reconstituted with a mixture of primary human nasal epithelial cells isolated from 14 different healthy donors. 8 generations of pool were produced though 13 years each generation bearing distinct donors. Steady state and stimulated parameters have been compared such as TEER, Cilia Beating Frequency, cytokine secretion (IL-8) release. Furthermore, infections capacity of Influenza (H1N), Rhinovirus (RV-A16), and Respiratory Syncytial Viruses (RSV-A). Furthermore, data have been compared with bronchial (MucilAir™-Bronchial-Pool) and small-airways (SmallAir™- Pool) pool and mono donor ALI.

Data suggest that pool versions of ALI keep similar morphology and functions across pool generations. Pooling leads to an average response from donors and keeps stable data through years. On the other hand, donor specificity using mono donor ALI is an advantage to compare disease states outcomes.

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Development of TTC values for inhalable substances

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The threshold of toxicological concern (TTC) concept defines limit values for substances with a known chemical structure in the absence of sufficient toxicity data. Below these derived exposure limit values (Cramer Classes), the risk to human health is considered negligible. The TTC concept was developed and evaluated for orally administered substances but is less evaluated for inhalable substances [1-3]. The project aims to derive TTC values for airborne compounds (inhalTTC).

As part of a detailed review process, a database (DB) was established consisting of high-quality repeated dose inhalation studies. For each study, the N(L)OAEC (no (lowest) observed adverse effect concentration) values per local/systemic effects were determined using a predefined criteria system. Data standardization was conducted to enable analysis and facilitate the training of subsequent machine learning (ML) approaches. This includes standardization to one unit value and the application of time assessment factors for non-chronic LOAEC values.

The current inhalTTC DB comprises 966 studies, with sub-chronic exposure (59%), followed by chronic (22.1%) and subacute (18.8%) exposures. Analysis shows no significant difference between the distribution of the representative local and systemic normalized chronic LOAELs stratified to study duration. A classification system is currently being developed to differentiate high and low toxic airborne substances and to identify factors influencing the level of toxicity using a variety of ML techniques. ADME and physiochemical properties will enter these models as potential influence factors. Initial ML results will be presented.

The inhalTTC DB and the ML are expected to allow the derivation of threshold values for airborne compounds based on their structural features and properties.

This abstract does not reflect EPA policy.

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Development of a human iPSC-derived kidney-on-a-chip cell model for the application of toxicity testing

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Introduction: Up to date, animal experiments are still the golden standard for toxicity testing. However, human-based *in vitro* models for toxicity testing are emerging. A challenge that is often observed in these *in vitro* models is that cells lose expression of important xenobiotic transporters, like organic anion transporters (OAT) and organic cation transporters (OCT). This complicates accurate assessment of drug-induced nephrotoxicity in renal *in vitro* models. In this study, we mono- or co-cultured human induced pluripotent stem cell (iPSC)-derived proximal tubular-like cells (PTL) with iPSC-derived endothelial-like cells (ELC) from the same donor in the TissUse HUMIMIC chip 3 organ-on-a-chip (OoC) system to assess OAT and OCT transport capacity in PTL compared to static cultures.

Methods: PTL and ELC were differentiated as previously described [1,2]. Three different conditions in the OoC were tested: Monoculture of PTL on filters, Co-cultures of PTL/ELC on filters and co-cultures of PTL on filter and ELC in a separate compartment in the OoC device. All conditions were cultured in the OoC for up to 3 weeks and compared to cells cultured on filters under static conditions. PTL and ELC marker expression (PTL: megalin and ZO3; ELC: CD-31) was assessed and barrier tightness was measured through transepithelial electrical resistance (TEER) and lucifer yellow assays. Functional OAT and OCT transport was assessed through active transport assays. Cell viability of compounds known to be transported via these transporters were assessed.

Results: PTL and ELC could be grown up to 3 weeks; ELC expressed CD-31 and PTL showed expression of megalin and ZO-3. TEER values of PTL increased within the first week of culture, after which they stabilized gradually within weeks 2 and 3. Lucifer yellow assays showed a tight barrier of PTL from day 21 to 27. Functional OCT was observed in PTL in all conditions; no significant differences were observed between static and chip cultures. In co-cultured PTL with ELC in chips, OAT transport increased on day 23 compared to day 16 and day 9. In PTL and ELC cultured on the same filter, a nearly 3-fold increase in OAT transport on day 23 was observed compared to day 16 and day 9. This suggests that the proximity of ELC to PTL seems to affect functional OAT transport capacity of PTL. Currently, assessments of changes in gene expression levels and cell toxicity studies are ongoing.

Conclusions: Our developed model shows improved transport function in the iPSC-derived kidney system and indicates a promising outlook for the application of nephrotoxicity testing.

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SmartMembrane: A next-generation *in vitro* cell culture platform featuring real-time, non-invasive cell analysis and AI-driven classification

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Objectives: Beginning in 2022, the FDA will no longer require animal experiments to validate drug candidates before human clinical trials, but reliable *in vitro* models are still essential for preliminary testing. Organ-like cell cultures (e.g., organoids) combined with microphysiological systems – known collectively as organ-on-chip – are a promising solution to replace or at least reduce the amount for animal testing. However, the high variability of such biological cell cultures and inadequate sensor technologies hinder the accurate evaluation of cell quality and drug effects. For example, TEER (transepithelial electrical resistance), the current gold standard, typically involves only a single frequency, is incompatible with simultaneous live-cell imaging, and is incompatible with air-liquid interfaces. To address these limitations, we developed SmartMembrane, a novel sensor that enhances measurement capabilities and thus improves *in vitro* drug testing.

Results: Our sensor employs co-planar microelectrodes (Ti/Au) on an ultrathin (700 nm) porous silicon nitride membrane. This design enables real-time monitoring of cell status – encompassing confluence, density, and type – using impedance spectroscopy, which behaves as an “electrical fingerprint.” A trained neural network then classifies these impedance spectra and accurately identifies the stage of barrier formation (from initial cell inoculation to a mature, dense layer) for both endothelial and epithelial cells. Moreover, our platform continuously tracked both reversible and irreversible cell damage, detecting subtle changes in tight junctions that were not evident in conventional endpoint analyses by immunostaining. Hence, even slight impairments to barrier function become measurable in real-time, well before they manifest in standard assays.

Conclusion: Our findings underscore that automated and noninvasive real-time monitoring of barrier dynamics is feasible in barrier-on-chip systems without reliance on microscopy or endpoint staining. We anticipate that the SmartMembrane platform will achieve broad applicability in organ-like models, facilitating precise monitoring of both physiological and pathological states in barrier tissues. By providing insightful, continuous data on tissue integrity and viability, SmartMembrane advances the reliability and efficiency of *in vitro* drug testing in line with emerging FDA guidelines.

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Innovation or adaptation? An integrative review of the advances and challenges of the new generation of biomarkers and methodologies in genetic toxicology

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The assessment of genotoxicity and mutagenicity is crucial to understanding the health risks caused by substances. This study reviews the main advances in assessment methodologies, identifying the limitations of traditional approaches and exploring the potential of new biomarkers and alternative methodologies (NAMs). The objective was to analyze the limitations of classical methodologies and investigate how new biomarkers can improve the predictability and applicability of alternative approaches. The research used the PICo model for qualitative studies, considering current assessment methodologies, new biomarkers as the phenomenon of interest and improved predictability as the context. The integrative review was carried out in the PubMed and Medline/PubMed databases, including primary articles published in the last five years in Portuguese, English or Spanish. Reviews, meta-analyses, out-of-scope studies and studies of low methodological quality were excluded. The selection and analysis of studies were performed in pairs and followed the PRISMA method, ensuring rigor in the evaluation. The initial search resulted in 767 articles in PubMed and 49 in Medline. After filters and screening, 102 articles from PubMed and 6 from Medline were selected. Most of the studies focused on the evaluation of genotoxicity, with only 1% addressing mutagenicity and carcinogenicity. In addition, few studies investigated new biomarkers and methodologies for toxicogenetics. It was observed that, instead of disruptive innovations, adaptations of traditional tests, such as the micronucleus test and the comet assay, applied to different experimental models, predominate. Regarding methodological approaches, 44% of the studies used *in vivo* models, 56% *in vitro* techniques and 5% *in situ*, reflecting the increasing replacement of the use of animals by more ethical methods. Among the new biomarkers, the use of transcriptomic biomarkers stood out due to their high sensitivity and ability to elucidate molecular mechanisms, in addition to their integration with omics technologies and artificial intelligence. There was also greater interest in biomarkers such as gamma H2AX, P53 and the Pig-A gene, which allow the early detection of genetic damage and are accepted as reliable biomarkers for the prediction of mutagenic and carcinogenic risks. It is concluded that, in the last five years, research in genetic toxicology has been, for the most part, incremental innovations, with adaptations of traditional methods to new experimental conditions, which is understandable, since these methods are already well known. Although there have been advances in the use of more sen-



sitive biomarkers, such as transcriptomics, disruptive innovations, capable of radically transforming the field, are still limited. The research also showed a growing replacement of the use of animal models by *in vitro* approaches, reflecting a trend towards more ethical and predictive methods.

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3D intestinal barrier models: A cutting-edge approach as alternative to animal testing in drug toxicity assays

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The main challenge in preclinical drug response studies is the limited complexity and predictive of conventional *in vitro* models, which fail to accurately replicate the structural and functional intricacies of native tissues. In this study, we generated three advanced 3D intestinal barrier models using biofabrication techniques to enhance physiological intestine relevance: (i) a manually model comprising intestinal epithelial cells (Caco-2/HT-29) cultured on top a collagen matrix; (ii) a more complex manual model integrating a Caco-2/HT-29 epithelial layer over a collagen matrix embedded with human dermal fibroblasts (HDFn); and (iii) an innovative bioprinted model incorporating both cellular layers. All models successfully recapitulated important intestinal barrier features, including tight junction formation (occludin), epithelial integrity (TEER), permeability (Dextran Alexa Fluor-488[®]), microvilli (H&E), and mucus production (mucin-2). Furthermore, all models exhibited up-regulation of genes associated with intestinal functionality during culture (Claudin-2, ABCB1, ATP1A1, and SLC5A1), confirming the tissue differentiation. After exposition to Ibuprofen, the bioprinted model demonstrated superior response accuracy, demonstrating its enhanced barrier functionality and physiological relevance. This study highlights the potential of 3D biofabricated models in replicating the native intestinal barrier, thereby improving the predictive accuracy of preclinical drug toxicity assessments while reducing reliance on experimental animal models.

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Enhancing induced pluripotent stem cell-derived liver organoids for advanced toxicity testing

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Introduction: Toxicity testing is essential in drug development to predict adverse effects in humans before clinical trials. Recent advancements in liver organoids derived from induced pluripotent stem cells (iPSCs) provide a promising alternative to animal models, which often fail to accurately predict human-specific responses and raise ethical concerns. However, current human liver organoid (hLO) models still face challenges related to maturity, functionality, and reproducibility, limiting their application in high-throughput toxicity testing. AIMS. To enhance the development and application of hLO by optimizing iPSCs differentiation protocols, incorporating advanced techniques, and employing different toxicity assays.

Materials and methods: We refined existing iPSC differentiation protocols and hLO models using xeno-free reagents, including replacing Matrigel[®] with a synthetic gel for both coating the iPSCs plates and the organoids' growth. To enhance maturation and metabolic activity, we supplemented the culture with short-chain fatty acids (SCFAs) and amino acids (AAs), which have been associated with improved *in vitro* liver maturation.

Results and discussion: hLOs differentiated on Laminin-511-coated plates showed no significant difference in albumin secretion compared to those on Matrigel[®]-coated plates. However, the addition of AAs and SCFAs led to an approximately fivefold increase in albumin secretion, as assessed by ELISA. Furthermore, SCFAs and AAs enhanced the fluorescence intensity of key hepatic markers, including CYP3A4, HNF4 α , and AFP, as well as the mature hepatic marker PEPCK, particularly in hLOs differentiated on Laminin-511-coated dishes. Additionally, markers for non-parenchymal cells were upregulated in the presence of AAs and SCFAs, including the biliary marker OPN, stellate cell marker Desmin, endothelial cell marker PECAM-1, and Kupffer cell marker CD68.

Conclusion: Our initial findings suggest that supplementing with AAs and SCFAs enhances the functionality of hLOs intended for toxicity testing. Additionally, Laminin-511 appears to be a viable alternative to Matrigel[®]-coated dishes. Overall, this approach has the potential to significantly improve the predictive power of *in vitro* toxicity testing, reduce reliance on animal models and animal-derived reagents, and accelerate the development of safer pharmaceuticals.

This Project received funding from Stiftung ProCare, Switzerland.

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Tebuconazole and pyraclostrobin: Ocular biodistribution assay vs. bovine corneal opacity and permeability test (BCOP)

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Introduction: A pesticide is any substance or mixture of substances intended to prevent, destroy, repel or mitigate any pest. Rural workers are one of the main groups subject to exposure to pesticides, since they are in contact with such products during transportation, mixing and application. It is already known that the most frequent routes of exposure to pesticides are inhalation and theoretical. However, the ocular route should be the object of attention, since, after contact of the substance with the ocular surface, permeation through the cornea and conjunctiva/sclera may occur.

Objective: To perform an ophthalmic biodistribution test, histological evaluation and opacity and permeability test in bovine corneas (BCOP) after exposure to the pesticides Tebuconazole (TBZ) and Pyraclostrobin (PIRA), using whole eyes.

Methods: Aqueous dispersions of Rival 200 EC® (Tebuconazole 200g/L) and Comet 250® (Pyraclostrobin 250 g/L) were prepared. For TBZ, the concentrations were 0.2 mg/mL and 0.4 mg/mL, and for PIRA, 0.3 mg/mL and 0.5 mg/mL. Whole eyes of cattle and pigs purchased

from a local slaughterhouse were used to assess biodistribution. 30 and 50 µL of the dispersions were applied to the surfaces of porcine and bovine eyes, respectively, and the system was maintained in an incubator at $32 \pm 1^\circ\text{C}$ and $65\% \pm 3$. In some specific details, aqueous humor was collected, and the eyes were excised to collect their internal structures (cornea, sclera, iris, vitreous humor, choroid and retina) for evaluation of biodistribution. For porcine eyes, the cornea, sclera, iris, vitreous humor and choroid were collected. In addition, histological evaluation of the corneas was performed to evaluate possible tissue damage. In addition, the (BCOP) was performed to evaluate the pesticide dispersions and their concentrated products.

Results: In the biodistribution test for bovine and swine eyes, when the lowest concentration was applied, there was an increase in permeation after 1 and 1.5 h for PIRA and TBZ, respectively. On the other hand, when the highest concentrations were applied for both products, there was an increase in the amount permeated. Regarding retention, greater amounts of pesticide were found in the cornea, followed by the iris, vitreous humor, choroid and retina. The application of higher concentrations also reflected greater retention in all structures evaluated. In the histological evaluation of the bovine and swine corneas, it was possible to observe changes in the epithelium, edema and disorganization of the stroma, demonstrating tissue damage. In the BCOP evaluation, the diluted products were classified as uncategorized, not being capable of causing serious damage to the cornea, and the concentrated products, as indeterminate, where it was not possible to make a prediction regarding the potential for ophthalmic advice.

Conclusion: The results demonstrate that the assessment of ocular biodistribution can be a useful alternative method in assessing the passage of different substances through the cornea, with BCOP being an auxiliary method in assessing the ocular barrier.

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Evaluation of the performance of amorolfine-based nail formulations

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Introduction: Onychomycosis is a chronic fungal infection of the nail. The effects of this infection, when left untreated, can include pain, secondary bacterial infections, and low self-esteem due to disfigurement of the affected nails. The nail is a resistant barrier that hinders the penetration of drugs, due to its composition and the presence of disulfide bridges that interconnect the keratin fibers. This barrier makes topical treatment more difficult and therefore the development of formulations becomes a challenge.

Objectives: To evaluate the performance of nail formulations based on Amorolfine (AMF), through the *in vitro* permeation test (IVPT) (OECD TG 428), using healthy human nails and additionally, through an adapted model with fungi.

Material and methods: Microemulsion (ME) formulations containing 5 and 7% AMF and thermogels (TG) with 5% active were developed and tested in the presence of different chemical promoters. In vertical diffusion cells, clippings of healthy human nails with pores that were formed with the aid of the Hydra.needle[®] device were used. The tests were conducted with a single dose of 50 µL. The nail collection was approved by the ethics committee of the Federal University of Pernambuco (CAAE: 27554719.1.0005208). Additionally, a histological evaluation of the nails was performed to evaluate the pores as well as the experimental model adapted for *in vitro* antifungal evaluation.

Results: In the experimental condition of single dose (50 µL) + poration, it was possible to demonstrate a greater retention from the ME 7% formulation. Comparing the retentions between the treatments, it was not possible to observe statistically significant differences between the formulations, even using different chemical promoters. Although the amount of drug that reached the receptor liquid was below the quantification limit of the analytical method (1 µg/mL), the permeated AMF was sufficient to generate inhibition of *C. albicans* and *C. parapsilosis* (presence of halo) from the adapted model. Microscopy helped to visualize the pores, which were uniform, acting as safe physical permeation promoters.

Conclusion: The IVPT results suggest that the combination of nail microporation using Hydra.needle[®] and a single dose favored nail permeation of AMF for all formulations. This demonstrates the importance of *in vitro* permeation studies in evaluating the performance of topical nail formulations. In addition, the adapted *in vitro* microbiological model confirmed the arrival of the active ingredient to the nail bed.

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Developing a hPSC-derived teratoma *in vitro* model for hPSC malignancy assessment

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Human pluripotent stem cells (hPSCs) have a myriad of potential clinical applications due to their capacity to self-renew and differentiate towards any of the embryonic germ layers. However, hPSC-derived cell products have shown to have the risk of tumor formation when used *in vivo*. To circumvent this issue, the safety of their use needs to be addressed, which includes the evaluation of the intrinsic hPSC malignant potential *in vitro*. Due to our limited knowledge on the underlying mechanisms causing this malignant potential, this assessment has traditionally been performed focusing on the histological traits of malignancy by xenografting the cells into immunodeficient mice (teratoma assay), for later evaluating the tissues found in the resulting tumor. Although informative, this assay faces scrutiny due to the lack of standardization of the procedure, the long waiting times and high related costs as well as the limited predicted value and questionable ethics behind animal testing. Here, we aimed at developing a reliable, standardized and fully animal-free *in vitro* model able to recapitulate the teratoma assay for addressing the histologic features of hPSC malignancy in an efficient manner. We generated embryoid bodies (EBs) as basic models of unbiased cell differentiation from validated malignant and safe stem cells and exposed them to xeno-free and xeno-derived culture conditions. These were then compared on the basis of their capacity to generate structures displaying tissues with the same histopathological traits as those observed upon *in vivo* xenografting. Immature, fully differentiated, and mixed tissues were obtained in EBs from malignant stem cells, safe hPSCs, and iPSCs with impaired differentiation, respectively, cultured in both xeno-derived and -free conditions, although with low efficiency. Our results suggest that it is possible to develop an *in vitro* system able to recapitulate (immature) teratoma formation using xeno-free differentiation conditions, although efficient tissue formation can be improved potentially by better mimicking the mouse injection site microenvironment through co-culture and hydrogel embedding conditions.

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Nanoencapsulation of amphotericin B: Toxicological findings that may indicate its increased bioavailability in zebrafish embryos

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Amphotericin B (AmB) is a commonly used antifungal agent, which is also prescribed for the treatment of certain neglected tropical diseases, including leishmaniasis. However, its clinical application is constrained by its poor oral bioavailability and adverse effects, prompting the investigation of alternative drug delivery systems. Polymeric nanoparticles (PNP) have gained attention as potential drug delivery systems, providing advantages such as sustained release and enhanced bioavailability. However, concerns persist regarding nanomaterials' toxicity, requiring more nanotoxicity studies. Zebrafish (*Danio rerio*) embryos have been used as a valuable model for toxicity testing, especially by their genetic similarity to humans and standardized developmental assessments. In this study, AmB-loaded poly(lactic acid) (PLA) and polycaprolactone (PCL) PNP were produced and tested *in vivo* with zebrafish embryos and larvae. Firstly, we demonstrated, by fluorescence microscopy, that PNPs penetrated the animals' bodies. Then, in toxicity tests adapted from the OECD TG 236, an increase in their toxicity was observed, especially when compared to non-nanoformulated AmB. However, it was previously demonstrated that the polymers used are safe and that they themselves are not the cause of toxicity. Regarding possible cardiotoxicity, PNP containing AmB were shown to be safe, in a heart rate counting assay of the tested larvae, performed at the last day of exposure. We also demonstrated that the number of hours post-fertilization prior to the start of the test influences the obtained results. In conclusion, we strongly believe that the objective of improving the bioavailability of AmB may have been achieved, as our microscopy and toxicological results suggest that there was an increase in the absorption of the drug by the zebrafish embryos.

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Pyrogenicity paradigm shift: Moving from rabbits to human-relevant research

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Efforts to develop ethical, human-centric methods for assessing health risks have grown, fostering opportunities to move away from animal testing. Despite this, nearly one million animals were used for regulatory testing in Europe from 2018-2022, with over half used for product quality control (QC). Batch safety/potency testing is a type of QC test where non-animal alternatives can offer potential, but adoption of these new models face historic, scientific, and regulatory barriers. However, these challenges are not limited to Europe as the shift to non-animal methods is a global endeavor.

Pyrogenicity testing exemplifies progress in overcoming these globally recognized challenges. Historically, this QC test was reliant on the Rabbit Pyrogen Test (RPT), but its ethical and scientific limitations spurred the development of *in vitro* alternatives like the Monocyte Activation Test (MAT). MAT is now validated and accepted in Europe, United States, Russia, and India, and recently, Brazil became added to this list as the Brazilian Pharmacopeia now includes MAT. However, so far, only the European Pharmacopeia (Ph. Eur.) has completely phased out RPT, meaning reference to the *in vivo* method has been removed from all monographs. This differs from countries like the US, where RPT is still considered the gold standard, and MAT is not considered compendial to RPT. Similarly, the Brazilian Pharmacopeia lists both tests, with ongoing efforts to harmonize with international standards through the advocacy of the Brazilian Centre for Validation of Alternative Methods (BRACVAM) for greater MAT adoption. Overall, this illustrates that MAT adoption remains inconsistent globally. Regulatory inertia and slow progress in phasing out RPT are highlighted as disparities.

This presentation analyzes the phase-in of MAT and RPT phase-out by examining scientific, historical, and regulatory factors. It highlights Europe's slow but steady shift, nearly 40 years in the making, and the US lag due to regulatory inertia. It also touches on Brazil's evolving regulatory landscape, where the inclusion of MAT in the pharmacopeia signals progress, but global implementation still faces barriers. We find that accelerating transitions requires parallel method phase-ins and phase-outs, proactive legislation prioritizing non-animal methods, and incentivized regulatory frameworks to make animal testing a true "last resort." By integrating insights from various perspectives, this analysis aims to foster a globally coordinated approach to ethical testing practices.

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Mouse suffering for vanity: Tracing replacement of the LD50 bioassay for botulinum neurotoxin

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Botulinum neurotoxin (BoNT) is widely used in medical and aesthetic applications across the globe. More specifically, BoNT use for aesthetic purposes is steadily rising in South America, with Brazil representing one of the largest markets for non-surgical treatments globally. As a biological product, BoNT requires rigorous batch potency testing to ensure safety and is currently reliant on the ethically and scientifically contentious mouse lethality bioassay (MLB). Despite severe suffering of the mice, the reproducibility crisis and translatability issues of this LD50 test, its use persists due to regulatory inertia and systemic barriers.

Recent advancements in new approach methodologies (NAMs), particularly cell-based assays, offer promising non-animal alternatives for final product testing. However, adoption is hindered by the lack of universal standards, product-specific variability, and the legislative necessity to validate NAMs against MLB data. This presentation explores the scientific and regulatory factors affecting the transition to non-animal NAMs with a primary focus on Europe as a key player in the production of BoNT products. Namely, it describes the (accepted) alternatives to the MLB, the distribution of BoNT products in Europe, mice use for potency testing in the European Union and United Kingdom and attempts to understand the number of mice used per year for BoNT alone by analyzing non-technical summary (NTS) data from known testing facilities across the UK, Germany, and Ireland.

This presentation aims to synthesize findings from publicly available resources to provide actionable insights into overcoming barriers to implementing ethical and scientifically robust alternatives for regulatory testing. Furthermore, it highlights the global nature of this issue, emphasizing that aligning regulatory frameworks – including in regions like Brazil – is essential for accelerating the transition to humane and innovative safety practices worldwide

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Advancing cosmetic safety: 3D spheroids for reproductive toxicity testing

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Reproductive toxicity can be categorized into two major areas: fertility, related to adults and referring to gamete quality, hormonal dysregulations, and defects in gametogenesis; and developmental toxicity and teratogenicity, which refer to embryos and fetuses and can lead to congenital anomalies, intrauterine growth restriction, embryonic or fetal death, and preterm birth. These effects can arise from a variety of causes, including chemical agents, pharmaceuticals, radiation exposure, environmental factors, nutritional deficiencies, and microbial organisms. Moreover, these effects can result from multiple routes of exposure, including topical application, which is particularly significant in the cosmetic industry. Conducting reproductive toxicity studies in cosmetics is essential due to the consumer's exposure to ingredients such as fragrance molecules and exclusive raw materials derived from biodiversity. Developing alternative methodologies for evaluating this endpoint is crucial for anticipating regulatory classifications, reducing reliance on animal testing, and ensuring product safety.

Therefore, we have developed and characterized ovarian and testicular spheroids as innovative *in vitro* alternative models for assessing reproductive toxicity. For ovarian spheroids model, we optimized parameters including cell seeding density (2 million cells *colocar o nome de célula* per mold) and incubation time (72 hours) in agarose micro molds. Cell viability was confirmed using controls with culture medium and cytotoxic treatments with DMSO (10% and 50%), a substance well-known for its cytotoxic effects on cells. The functional assays were performed using bisphenol A (BPA), a well-known reproductive toxicant that leaches from a variety of everyday products, including plastic containers for food and water, as well as packaging for infant products. A concentration of 200 μ M, selected based on transcriptomic changes that do not induce significant cytotoxicity, activated the estrogen receptor pathway, suggesting potential endocrine disruption. Additionally, alterations were observed in markers related to the development and function of the endocrine system.

A similar optimization was conducted for testicular spheroids, with the optimal conditions achieved using 2 million cells per mold incubated for 24 hours. Viability tests demonstrated sensitivity to DMSO. Functional tests involved exposing the cells to 200 μ M diethyl phthalate, a substance recognized for its potential to induce testicular toxicity. The key findings included a downregulation of SUMOylation pathways and retinoic acid receptor signaling, both of which are essential for testicular differentiation and maturation. Upregulated pathways included aryl hydrocarbon receptor (AhR) and Wnt/ β -catenin, indicative of cellular stress responses. The findings emphasize the value of ovarian and testicular spheroids as effective predictive instruments for assessing reproductive toxicity, providing reliable models for evaluating cosmetic safety while decreasing dependence on animal experimentation.

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3D models of hepatic spheroids for toxicity testing: Training and empowerment of future scientists

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The present project, conducted at the Tissue Engineering Laboratory of LNBio/CNPem, aimed to validate a toxicity test using hepatic spheroids as an alternative to animal testing. The study was carried out by high school students from public schools in the State of São Paulo, participating in the Future Scientists Program of CETENE/CNPq. Over four weeks, the students received theoretical and practical training in biosafety, cell culture techniques (e.g., 3D culture models), and cell analysis (e.g., toxicity testing). In addition to technical-scientific training, ethical issues related to scientific research and the 3Rs principle were addressed, providing a critical perspective on biomedical research. Hepatic spheroids were obtained from HHStEC and C3A cells using agarose micromolds for biofabrication. The toxicity of widely used chemical compounds – acetaminophen, acetylsalicylic acid, and formaldehyde – was tested at two concentrations, and cell viability was assessed using the MTT assay. The results indicated that formaldehyde exhibited high toxicity, aligning with its classification as a hazardous substance by the WHO and its ban by ANVISA. Acetylsalicylic acid showed significant toxicity at high concentrations, suggesting potential liver damage, while acetaminophen displayed lower toxicity, reinforcing its safety when used in controlled doses. Beyond the scientific findings, one of the project's main impacts was the academic and personal development of the students. The experience enabled them to develop technical and analytical skills, leading to data interpretation and the presentation of the study at a national level. Exposure to the research environment broadened their professional perspectives, sparking interest in scientific and technological careers. Thus, in addition to contributing to the dissemination of alternative methods to animal testing, the project fostered the training of future scientists and encouraged female participation in research and technological innovation.

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Using the experimental design assistant and the ARRIVE guidelines in teaching to improve implementation of the 3Rs

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This presentation will help those with teaching roles support the reduction “R” of animal use. The focus will be on how to use existing free-to-use resources to teach researchers about rigorous experimental design, which is important for ensuring animal experiments deliver reliable results and minimizes the risk of wasting animals in poorly designed studies. The presentation will highlight how the NC3Rs’ Experimental Design Assistant (EDA) and ARRIVE guidelines can be used in teaching. The EDA is a free-to-use online tool that helps researchers design experiments rigorously and communicate them effectively. This presentation will show how the EDA can be integrated into undergraduate teaching to ensure knowledge of experimental design and its importance for the next generation of scientists. It will also show how a train-the-trainers approach can be used with the EDA to help institutions train researchers to design more rigorous experiments. The presentation will also cover how ARRIVE guidelines resources, such as the ARRIVE manuscript review exercises, can be used in teaching to help students learn to assess whether there is enough information in an animal research paper to tell if the results are likely to be reliable.

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Full-thickness 3D skin co-culture with immune cells: A novel model for transcriptomic analysis of dermal sensitization

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Introduction: The evolution of alternative methods for safety assessment has been constantly pursued since more robust answers have been necessary to evaluate toxicological outcomes, as well as to evaluate substances of varied origins and compositions. In the cosmetic industry, evaluation of sensitization endpoints helps in the development of safe formulations to the consumers. Currently, the potential dermal sensitization is assessed by *in vitro* tests covering, independently, each key event of Dermal Sensitization pathway (OECD 442C, 442D and 442E). Using a full-thickness 3D skin model co-cultured with immune cells makes it possible to evaluate the dermal sensitizing potential with simultaneous analysis of two key events (activation of skin keratinocytes and immune cells response) and the presence of a dermal layer with fibroblasts suggests a more robust response regarding cell-cell communication.

Objective: Enable an immunocompetent full-thickness 3D skin model to evaluate the potential of dermal sensitization of substances with cosmetic application.

Materials and methods: The full-thickness 3D skin model was grown in co-culture with human monocytes (THP-1). To assess sensitizing responsiveness, co-cultures were exposed to proficiency substances applied to the skin. A transcriptomics analysis was performed to evaluate gene expression regarding activation of both keratinocytes and immune system cells. The transcriptomics method performed was real time PCR using a customized panel with selected relevant markers and the modulation of interest biological pathways was assessed by the specialized software IPA (Ingenuity Pathway Analysis – QIAGEN).

Results: The exposure to proficiency substances, as 1-chloro-2,4-dinitrobenzene (DNCB) and Cinnamaldehyde, resulted in the stimulation of genes related to dermal sensitization regarding keratinocyte and monocyte activation represented by pro-inflammatory cytokines, NRF2 signaling, dendritic cell maturation and other pathways of interest.

Conclusion: The use of an immunocompetent full-thickness 3D skin model showed promising potential for dermal sensitization assessment. Integrating two key events into one system offers a more robust and efficient analysis of dermal sensitization, contributing to the improvement of safety assessment strategies in the cosmetic industry.

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Addressing reproducibility challenges in scientific research: A comparative analysis of scratch assay protocols using artificial intelligence

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Test reproducibility has been a persistent challenge in scientific research worldwide. In recent years, AI-based tools have gained prominence in optimizing scientific processes, particularly in enhancing reproducibility. PubCompare, a tool designed to address this issue, identifies and compares experimental methodologies in the literature. It allows researchers to input keywords or protocols to locate similar studies and analyze variations in procedures. By facilitating the selection of more robust methodologies, PubCompare aims to support research with greater reproducibility and reliability. It strives to be a key tool for achieving reproducibility, helping scientists save time, reduce waste, and produce better results.

The issue of reproducibility extends to *in vitro* tests, which assess cellular responses to various products and present a critical hole in alternative methods, particularly in efforts to reduce animal use in experimentation. One commonly used test is the Scratch assay, which involves plating cells, expanding them to high confluence, creating a scratch, applying the test substance, and evaluating cell growth to close the scratch. However, the literature reveals a wide range of protocols for this test, with no single consensus in the scientific community. This lack of standardization can lead to inconsistent results, even when the same test is supposedly replicated.

In this study, PubCompare was used to evaluate the main differences between Scratch assay protocols described in five articles published in 2017 (n = 1), 2022 (n = 2), and 2024 (n = 2). Inclusion criteria were open-access publications, use of HaCaT cells, and performance of the assay in a 6-well plate. Steps compared included cell seeding, scratch wound creation, imaging time points, and image analysis.

Results revealed significant variability in protocol details. For cell seeding, three articles did not specify the initial number of cells seeded, while one used 1×10^5 cells/mL and another used 2×10^5 cells/mL. Regarding scratch wound creation, all protocols used pipette tips, with four out of five citing a volume of 200 μ L. Imaging time points varied, with two protocols analyzing at 0 and 24 hours, one at 12 hours, and two at 24 hours post-scratch. Image analysis software differed, with ImageJ used in two protocols, NIH Image in two, and one protocol not specifying the software used.

These findings highlight a lack of standardization in Scratch assay protocols and the omission of crucial details necessary for reproducibility. Key aspects, such as initial cell count, pipette volume for scratch creation, and post-scratch analysis time points, were inconsistently reported. This variability underscores the broader reproducibility crisis in scientific research. A previous study involving 1,576 researchers found



that 52% acknowledged issues with reproducibility, and nearly 90% emphasized the need for “more robust experimental design” as a critical factor for improvement.

The lack of detailed information in published articles poses a significant barrier to replicating experiments. As demonstrated in this study, researchers must make a concerted effort to improve the quality of data reporting and the reproducibility of experimental protocols. Tools using AI can play a pivotal role in addressing these challenges by promoting transparency, standardization, and robust methodology in scientific research.

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Development of a placental barrier model for reproductive toxicity assessment

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Reproductive toxicity is defined as adverse effects on the reproductive system, which can be divided into two main areas: fertility toxicity and developmental toxicity. Fertility toxicity primarily affects adults, potentially leading to impaired production of gametes, hormonal imbalances, or abnormal gametogenesis. On the other hand, developmental toxicity and teratogenicity are related to embryos and fetuses and result in congenital anomalies, intrauterine growth restrictions, embryonic or fetal

death, and premature birth. Studying reproductive toxicity is essential to understanding and mitigating the risks posed by these factors, particularly in contexts like the cosmetic industry, where safety and regulatory compliance are paramount.

A crucial aspect of developmental toxicity is the placental barrier, a physiological membrane regulating the two-way traffic of substances between the maternal organism and the developing fetus. This barrier is important in reducing the exposure of the fetus to potentially harmful substances while providing for the passage of essential nutrients. From a structural aspect, the placental villi are composed of syncytiotrophoblast cells and endothelial cells at the maternal and fetal interfaces, respectively, separated by a basal lamina. During early gestation, cytotrophoblast cells also contribute to the maternal interface. Replicating this complex barrier is essential for realistic teratogenicity assessments, as it closely mimics *in vivo* human exposure conditions. When combined with other organ-equivalent models, such as 3D skin, this model enables the simulation of specific exposure scenarios, including dermal chemical exposure.

To address this need, we developed an *in vitro* placental barrier model to simulate the maternal-fetal interface. To represent the maternal side, JEG-3 cells, which are epithelial-like cells taken from placental tissue were used. Human umbilical vein endothelial cells (HUVEC) were used to represent the fetal side. A layer of collagen was added to create an interface between the two types of cells, making up the extracellular matrix of the placental barrier. To validate the model, the barrier was exposed to 50% DMSO to assess cytotoxic responsiveness. Furthermore, the morphology was evaluated, as well as the Trans-epithelial resistance (TEER) and the permeation capacity of this placental barrier. The present model is a physiologically relevant tool for the assessment of chemical teratogenicity, especially under dermal exposure-simulating conditions. This approach provides robust predictive capacity of *in vitro* systems by reproducing major characteristics of the placental barrier but, at the same time, reduces reliance on animal testing.

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SenScreen: A new approach methodology combining full-thickness 3D skin and transcriptomics to assess skin sensitizers

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Skin sensitization is one of the most well-known toxicological endpoints, with an established adverse outcome pathway and validated *in vitro* methodologies covering each key event of the process. Nevertheless, the validated methodology used to assess the second key event, i.e., the activation of keratinocytes, named KeratinoSens and described in OECD test guideline 442D, is still expensive, mainly for underdeveloped countries. Thus, the development of new options of methodologies to assess this key event is necessary to expand the use of NAMs to assess skin sensitization.

We developed a new approach methodology to assess keratinocyte activation through the modulation of a gene panel which encompasses the main biomarkers involved in this key event. This gene signature is composed of four endogenous genes as control and 92 other genes whose products act directly on major pathways related to keratinocyte activation.

To validate the usefulness of this model, we tested proficiency substances known for their sensitizing potential, which were previously tested in OECD test guidelines for this toxicological endpoint, as 1-chloro-2,4-dinitrobenzene (DNCB), an extreme skin sensitizer, and Cinnamaldehyde, a weak skin sensitizer.

Our results show that this model is a promising tool to assess skin sensitization, in combination with other methodologies in an integrated approach.

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Lung-derived 3D spheroids: A cost-effective model for investigating SARS-CoV-2 infection and drug screening

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the COVID-19 pandemic. The World Health Organization (WHO) initially designated the outbreak as a Public Health Emergency of International Concern (PHEIC) and, within a month of its onset, declared it a pandemic. The rapid spread and continuous antigenic evolution of SARS-CoV-2 variants present ongoing challenges to global public health. Extensive investigations have provided critical insights into the virus's molecular biology, cellular tropism, and pathogenic mechanisms. However, *in vitro* models still face limitations in replicating the full spectrum of the infection process and assessing therapeutic approaches. Consequently, three-dimensional (3D) culture systems have emerged as an essential tool bridging the gap between traditional 2D cultures and *in vivo* models. Lung-derived spheroids, in particular, have been identified as a promising platform for studying SARS-CoV-2, as they more accurately mimic the physiological 3D microenvironment and offer a reliable model for drug screening. This study aimed to develop and characterize lung-derived coculture spheroids composed of epithelial and stromal cells and to investigate the impact of SARS-CoV-2 infection within this 3D model. Initially, 3D lung spheroids were formed by co-culturing human alveolar or bronchial epithelial cells with human lung stromal cells. Their morphology, size, and ultrastructure were evaluated before and after SARS-CoV-2 infection through optical and electron microscopy. The presence of the virus within the spheroids was confirmed by detecting the spike protein using immunohistochemistry. In addition, multiplex analysis was conducted to assess the cytokine release triggered by the infection. The spheroids remained stable, maintaining their size and morphology after SARS-CoV-2 infection, despite the presence of multivesicular bodies, endoplasmic reticulum rearrangement, tubular compartment-enclosed vesicles, and accumulated viral particles. In response to infection, the spheroids released IL-6 and IL-8 cytokines. This study highlights that cocultured spheroids of epithelial and stromal cells offer a cost-effective and physiologically representative model for SARS-CoV-2 infection. Due to their capacity to mimic essential features of the host response, we propose utilizing these 3D spheroids as a platform for drug screening to explore therapeutic approaches aimed at modulating cytokine release, with potential applications for treating long COVID (doi:10.1111/aor.14729).

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The potential use of a 3D skin model for senotherapeutics efficacy assessment

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Cellular senescence refers to the changes that occur in the body due to physiological processes and is a crucial mechanism that prevents tumor and tissue damage. While the immune system typically eliminates senescent cells, age-related decline in this function leads to their accumulation within tissues. Although senescence itself is not a disease, senescent cells in tissues can, paradoxically, contribute to pathological conditions. The persistence of senescent cells during aging induces chronic inflammation and tissue dysfunction through the senescence-associated secretory phenotype (SASP). Senotherapeutics are an emerging class of compounds that can assist the immune system in mitigating the effects of senescent cells in age-related disorders. This investigation sought to assess the potential use of a 3D skin model in cellular senescence studies. A senescent 3D skin model, composed of a viable epidermis equivalent attached to a dermis equivalent composed of 30% human senescent fibroblasts from a premature senescence process induced by UVB radiation, was used to assess the reduction of the characteristic SASP phenotype. Using two commercial molecules, quercetin and rapamycin, and a real-time RT-PCR assay, we assessed the potential of the ingredients to prevent senescence. Analysis of the senescent 3D skin differential gene expression revealed an increase in the activation of the main mechanisms related to senescence, when compared to non-senescent skin. Simultaneously, there was a reduced activation of mechanisms related to cellular physiological processes, such as extracellular matrix synthesis. Following the topical application of the senotherapeutic to the 3D skin, both samples showed improvement of gene expression response, when compared to the senescent skin control. Regarding interleukin signaling, all samples were able to reduce IL6 signaling and increase the activation of IL10 signaling, a known anti-inflammatory interleukin. When considering the senescence pathway, the Quercetin sample displayed a gene profile result similar to non-senescent skin sample, under the evaluated conditions. The data also suggest an influence on the inhibition of IL8 signaling activation and extracellular matrix degradation, while also indicating an increase in PPAR signaling. The PPAR family acts as transcriptional regulators, and changes in the transcriptome indicate influence on cutaneous inflammatory signaling. In conclusion, this study demonstrates that the 3D skin model can be used as a platform for evaluating the inhibition of the senescence phenotype, offering an alternative to animal models. Both quercetin and rapamycin demonstrated efficacy by reducing the signaling of degradation of the extracellular matrix when compared to senescent skin. Regarding the senescence pathway, the quercetin-treated sample was able to reduce the signaling related to cellular senescence, displaying a gene expression profile result similar to the non-senescent 3D skin sample.

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Development of liver spheroids as a biomimetic platform for drug testing and hepatotoxicity assessment

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The development of *in vitro* liver models that mimic native hepatic metabolism is essential for pharmaceutical applications, particularly for drug testing and hepatotoxicity assessment. Traditional models relying on animal testing often fail to accurately predict human responses, highlighting the need for advanced biomimetic systems. This study aims to develop liver spheroids with metabolic responses comparable to those of the native liver, providing an alternative platform for evaluating drug metabolism and toxicity. Liver organoids were generated using micromolded agarose hydrogel with 256 recesses, in which 5×10^5 cells were seeded. The co-culture approach included human hepatic stellate/endothelial cells (HUCLS/EA.hy926) and human parenchymal cells (hepatocytes – HEPG2). Morphological characterization was performed via hematoxylin and eosin (HE) staining, immunohistochemistry, and sphericity measurement. Functional analysis was conducted by quantifying Cytochrome P450 CYP3A4 activity, a key enzyme involved in the metabolism of drugs, toxins, food, and xenobiotics. Spheroids were treated with two concentrations of Dexamethasone (10 μ M and 100 μ M) to assess metabolic activity. The engineered liver spheroids maintained structural integrity after 11 days of culture, with an average sphericity of 0.87. HE analysis revealed round-shaped cells in the core and fibroblast-like cells at the periphery. Immunohistochemical evaluation demonstrated the presence of hepatic cells in the inner region (CK18+), endothelial cells at the outer border (CD31+), and stellate cells at the periphery (Vimentin+). CYP3A4 analysis indicated a 16.31% (10 μ M) and 43.70% (100 μ M) increase in enzymatic activity compared to the untreated control, confirming the spheroids' ability to metabolize drugs close to *in vivo* liver function. The results indicate that the development of liver spheroids through the co-culture of hepatic, stellate, and endothelial cells represents a promising approach for generating *in vitro* biomimetic liver. These spheroids offer a viable platform for *in vitro* drug testing and hepatotoxicity evaluation, addressing the limitations of traditional models. Future studies will focus on optimizing the model and further assessing its functional properties, including albumin, urea, and ALT/AST, as well as testing drugs classified by the FDA through DILIrank. All experiments are conducted based on the OECD guidelines for hepatotoxicity assessment in animals (OECD TG 452 and 408), ensuring alignment with internationally recognized standards.

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AI consensus model: A powerful tool for *in silico* mutagenicity screening of botanical extracts

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Botanical compositions consist of complex mixtures of natural products and are widely used in cosmetics. One of the major challenges in the safety assessment of these ingredients is the generation of toxicological data using New Approach Methodologies (NAMs). *In silico* methods, such as Quantitative Structure-Activity Relationship (QSAR) models, can be used in the preliminary stage of safety assessment to better guide the necessary *in vitro* methods. However, existing QSAR models for predicting compound toxicity have limitations in evaluating natural products, mainly due to the chemical space of these models. In this context, the development of new QSAR models with a high coverage of natural products in their chemical space can lead to more accurate predictions. A previously developed AI Consensus model, leveraging LightGBM, Support Vector Machines, and Random Forest algorithms with Extended Connectivity Fingerprints (ECFP), aimed to address this challenge by improving chemical space coverage for natural products. This study demonstrates the model's utility in safety screening by predicting the *in silico* mutagenicity of various botanical extracts and comparing these predictions with corresponding *in vitro* Ames test results. The mutagenic extracts evaluated were selected from scientific literature, where both phytochemical characterization and Ames test results were reported. These studies employed various analytical laboratory techniques to identify and quantify the key natural products present in the extracts, effectively determining the principal biological markers for each extract. The literature data also included at least one positive mutagenic result across various *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535, TA1537, TA97) and *Escherichia coli* WP2uvrA, with or without metabolic activation. Using the identified natural products within each extract *in silico* mutagenicity predictions were generated. These predictions successfully identified the mutagenic potential of most extracts, even with incomplete phytochemical characterization. This comparison between *in silico* predictions and *in vitro* Ames test results demonstrates that the AI Consensus model is a valuable tool for prioritizing and guiding further safety evaluations of botanical extracts. The model's ability to predict mutagenicity, even with limited chemical characterization, suggests its potential for broader application in the safety assessment of complex botanical mixtures.

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Adipose tissue organoids modulate epidermal regeneration in a 3D co-culture model

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Fat grafting is widely used for skin repair, however, the cellular and molecular mechanisms underlying its regenerative effects remain unclear. We hypothesize that skin regeneration is driven by the secretory activity of the stem cells population present in transplanted adipose tissue. To investigate this, we explored the feasibility of using fat organoids as a hypodermis component in bioengineered skin models. A 3D co-culture using SkinEthic™ Reconstructed Human Epidermis (RHE) with adipose tissue organoids was established following three experimental groups: RHE untreated control, Triton-treated RHE, Triton-treated RHE co-cultured with adipose tissue organoids. Adipose tissue organoids were generated by adipogenic induction of adipose stem cells (commercial lineage) spheroids for 15 days. The hypodermis was simulated by seeding (486 organoids/well). Co-cultures were maintained for 3 days in supplemented SGM medium. Adipose tissue organoids successfully fused within 3 days reaching a mimetic hypodermis. Fluorescence imaging confirmed lipid droplet formation, indicating adipogenic differentiation. Skin morphology analysis revealed that adipose tissue organoids positively modulated RHE regeneration compared to other groups. Cytokine profiling showed that key soluble factors (IFN- γ , GM-CSF, TNF- α , RANTES, VEGF, IL-8, IL-10) were downregulated in damaged RHE but restored in the presence of adipose tissue organoids. Notably, IL-10 and VEGF exhibited a positive modulation in treated RHE co-cultured with organoids. Our findings demonstrate that adipose tissue organoids can serve as a viable regenerative model resembling hypodermis and partially restoring RHE structure through the modulation of regenerative cytokines. While promising, long-term culture may be necessary to achieve full skin regeneration. These results highlight the potential of adipose tissue organoids not only as clinical substitutes for fat grafts and skin regeneration, but also as a physiologically relevant hypodermis model.

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Development and application of a microphysiological system as a BBB model for the study of neurodegenerative diseases and drug delivery

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2D cell culture is widely used for *in vitro* studies. However, it lacks accuracy and representativeness regarding cell-cell and cell-matrix interactions, which affects the assessment of drug responses and disease modeling. Although animal models provide physiological complexity, they often lack scientific validity for human diseases, as findings sometimes fail to translate accurately to human outcomes. In contrast, microphysiological systems (MPS), also known as organ-on-a-chip, offer advanced *in vitro* models that can replicate complex biological systems and barriers. The use of MPS in pharmaceutical and biomedical research is growing, in alignment with global initiatives and regulatory guidelines that support the replacement of animal testing with alternative methodologies. The xerography technique is a rapid and cost-effective method for producing microfluidic devices (MD) of MPS, facilitating the quick fabrication of molds and serving as an alternative to traditional techniques (e.g., soft lithography). This research aimed to create an MD through xerography, featuring a design that includes two side channels for medium flow and endothelial cell growth, along with a central channel where a hydrogel barrier is established. We evaluated

various hydrogels: gelatin methacrylate (GelMA) at 5% and 10% (w/V), either alone or in a 50:50 ratio with commercial extracellular matrix (Geltrex[®]) or bacterial nanocellulose (with or without reticulation using CaCl₂), identified as GE5, GE10; GE5_GX, GE10_GX; GE5_CB_CaCl₂, GE10_CB_CaCl₂; GE5_CB, and GE10_CB, respectively. They were assessed for mechanical properties (syneresis, strength, and stiffness) and cell viability of a glioblastoma cell line (U-87 MG in hydrogel), aiming to construct an *in vitro* blood-brain barrier (BBB) model. The hydrogels were integrated into the MD, and rhodamine perfusion was conducted to assess the perfusion rate. Swelling evaluated over 14 days indicated that up to the 7th day, hydrogels did not show statistical differences in swelling. However, after this period, hydrogels with Geltrex[®] expanded and ruptured the networks, with some dissolving before 14 days. The rheological results indicated that the compressive modulus revealed hydrogels prepared with GE10 had greater strength (300 to 800 kPa) compared to those with GE5 (150 to 400 kPa), except for those constructed with GX, which showed no significant difference in strength, approximately 300 kPa for both GE5_GX and GE10_GX. Young's modulus demonstrated that all hydrogels exhibited low stiffness (< 1 kPa), as expected for a BBB model in a physiological state. The stiffest hydrogels were those with GE10. U-87 MG cells remained viable after 14 days in both GE5 and GE5_GX but failed to survive in GE5_CB_CaCl₂ and GE5_CB. The permeation rate of rhodamine, a fluorescent small molecule, showed no resistance to permeation through the hydrogels in less than 30 min, with a flow rate of 1 µL/min. These primary results indicate the potential use of the MD as a BBB model for evaluating drug delivery system perfusion across the barrier. Further assessment of cytokines and other markers will facilitate the evaluation of neuroinflammation and anti-inflammatory effects of drugs. Additionally, validating this MPS will provide a biological model that has the potential to offer valuable insights into treatments for neurodegenerative diseases, thus reducing time and costs associated with preclinical drug screening studies.

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ASC spheroid-based 3D obesity model: A cutting-edge platform for investigating weight loss and inflammation

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Obesity is a complex metabolic disorder characterized by excessive adipose tissue accumulation and chronic low-grade inflammation. Advanced *in vitro* models that mimic the microenvironment of adipose tissue are crucial for studying obesity-related mechanisms and testing new therapeutic strategies. Here, we present a 3D obesity model based on adipose-derived stem cell (ASC) spheroids, evaluating their adipogenic differentiation, lipolytic response, cytokine secretion profile, and fusion capacity as a strategy to better replicate native adipose tissue. Abdominal superficial adipose tissue (SAT) was obtained from patients undergoing aesthetic plastic surgery. ASCs were isolated immediately after excision and expanded in monolayer culture until the second passage. ASC spheroids were then formed and subjected to adipogenic induction, cytokine analysis, and fusion assays. Lipolytic activity was assessed under LPS stimulation, and cytokine secretion was quantified after spheroid fusion. ASC spheroids demonstrated high adipogenic differentiation capacity in SAT. Under LPS stimulation, spheroids significantly increased lipolysis ($p = 0.0045$). They exhibited high synthesis of angiogenic cytokines, including VEGF, IL-6, and TNF- α . After 72 hours, spheroids underwent complete fusion, which markedly enhanced cytokine secretion. IL-6 and MCP-1 levels exceeded 40,000 pg/mL, while IL-8, VEGF, and IP-10 surpassed 20,000 pg/mL. Other cytokines (e.g., IFN- γ , GM-CSF, TNF- α , RANTES, IL-10, PDGF) were detected at lower levels ($< 20,000$ pg/mL). Notably, cytokine levels in fused organoids were at least 10 times higher than in non-fused spheroids. Moreover, fused organoids achieved a more advanced stage of adipogenic maturation, with predominant unilocular lipid accumulation. The ASC spheroid-based 3D obesity model successfully recapitulates key features of adipose tissue, including adipogenic differentiation, inflammatory response, and enhanced cytokine secretion following fusion. This model represents a promising platform for investigating obesity-related mechanisms and offers a relevant tool for *in vitro* drug testing in metabolic disorders.

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Generation of historical control database, acceptance and interpretation criteria in a comparative T4-glucuronidation assay using primary human and rat hepatocytes (TruVivo®) for regulatory acceptance

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There are no currently *in vitro* methodologies available with established guidelines, acceptance and interpretation criteria that can quantitatively evaluate liver-mediated thyroid hormone disruption across species to inform human relevance of chemically induced thyroid toxicity. Therefore, a need for New Approach Methodologies (NAMs) incorporating relevant test species and humans is crucial for safety assessment of chemicals. Additionally, a robust and reliable assay is key for regulatory acceptance. Current study is part of a method development, focused on evaluating the quantitative variance of T4 glucuronide (T4G) in rat and human hepatocytes after exposure to known nuclear receptor agonist reference compounds (NR-RFs).

Primary hepatocytes from 6 individual human donors (3 Male/3 Female) and 3 pooled Sprague Dawley rat lots (2 Male/1 Female) previously qualified were cultured in TruVivo® system. Cultures were exposed daily to NR-RFs: Polychlorinated biphenyl-153 (PCB153; 30 μ M), Pregnenolone 16 α -carbonitrile (PCN; 20 μ M), Phenobarbital (PB; 500 μ M) and Rifampicin (RIF; 10 μ M) at non-toxic concentrations for 7 days. Physiologically relevant T4 (0.05, 0.1 μ M rat & human) were added with 7th exposure. T4G was quantified in medium 24 hours post-T4 addition using a validated LC-MS/MS method. The data was statistically evaluated after three independent repeats with the objective of determining source of variation by variance component analysis (VCA) and generating historical control database (HCD) for control limits (CL)/acceptance criteria using Boot strap sampling. Furthermore, to establish assay interpretation criteria, 6 pharmaceutical compounds with preclinical & clinical evidence of T4 reduction, and increased induction of UGT in rodent studies (lansoprazole, carbamazepine, and phenytoin) or data showing no such evidence (levetiracetam, lamotrigine, and loratadine) were used to measure the T4G response in the aforementioned test system & acceptance criteria.

Vehicle control (VC) T4G data showed that rats have higher levels of T4G (range from 11 to 64.6 pmol/106/24 h) compared to humans (0 to 6.1 pmol/106/24 h). Reference compound induction (Δ T4G) by PCB153 was increased in both rat and human (range from 26.6 to 111 and 2 to 75 pmol/106/24 h, respectively). While PB induced Δ T4G in both species but much lower in human compared to rat. PCN (rat-specific) induced Δ T4G range from -3 to 34.7 and 0 to 1.8 pmol/106/24 h in rat and human respectively. RIF induced Δ T4G range from -16.4 to 24.5 and -0.3 to 5.8 pmol/106/24 h in rat and human respectively. Overall, a quantitative difference among the species was noticed and CL on VC and a positive control (PCB153) was established. VCA analysis confirmed inherit



variability majorly due to species (35-92%) and lots (2-50%). Further statistical work is in progress to establish the range of T4G response from the 6 pharmaceutical compounds to determine the range of positive and no response to define the assay interpretation criteria. Establishing a positive control, HCD, CLs, and acceptance criteria for the assay demonstrated its reliability, robustness, and reproducibility. Ongoing work aims to set interpretation criteria for the TruVivo® assay, in order to be acceptable to be used for predicting human safety of chemicals for regulatory submissions.

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Histone H2AX phosphorylation (γ -H2AX) assay as an alternative method for genotoxicity screening in the cytotoxicity bioassay platform at Fiocruz's network of technological platforms

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The Fiocruz's Network Technological Platforms help toxicology research through its Bioassays in Alternative Methods in Cytotoxicity facility, which offer *in vitro* cytotoxicity assays to predict compound toxicity. In 2024, over 500 analyses were performed for more than 200 researchers from public and private institutions. The platform offered alternative methods to reduce animal testing, aligned with Brazilian government guidelines. The facility also works on technological development and innovation in alternative methods to establish tests with greater

predictive capacity for human health. Currently, one platform objective is the development of tests to evaluate the compounds' genotoxicity. Genotoxicity assays are essential for identifying compounds that cause DNA damage, but traditional methods have limitations. The Ames test assesses only bacterial mutagenicity; the micronucleus assay detects chromosomal damage but does not distinguish between clastogenic and aneugenic effects; the comet assay is sensitive but lacks standardization. In this context, we are implementing a high-content screening (HCS) assay based on histone H2AX phosphorylation (γ -H2AX), a sensitive biomarker for DNA double-strand breaks (DSBs). Unlike traditional methods, γ -H2AX detects early DNA damage events. For standardization, we used γ -H2AX Rabbit Polyclonal Antibody (A300081A – Thermo Fisher®) and AlexaFluor-488 donkey anti-rabbit IgG (H+L) (A21206 – Invitrogen®). Etoposide (E1383 – Sigma-Aldrich®), a known genotoxic agent, was used as the positive control. The analysis is performed on the Operetta CLS™ system (PerkinElmer®) that provided precise quantification of γ -H2AX foci, allowing efficient data analysis. Etoposide induced γ -H2AX phosphorylation at 100, 50, and 25 μ M, confirming its genotoxic action. Our results suggest 25 μ M as the optimal positive control concentration, balancing effective DNA damage induction with minimal cytotoxicity. Compared to traditional assays, this HCS-based method offers faster and more sensitive. As a perspective, we intend to standardize the assay using ultraviolet (UV) light irradiation to induce DNA damage and reduce the genotoxic agents use in the scientific services offered by the platform. As a conclusion, this method presents advantages: Higher sensitivity because detects early DNA damage, unlike the micronucleus assay, which requires cell cycle progression; More specific, differentiates DSBs from other damage, unlike the comet assay; Automated and high-throughput, provides quantitative analysis; Mammalian-relevant, unlike the Ames test applies to human cells; Reduces chemical genotoxic agents, UV-based standardization may replace chemical inducers, enhancing sustainability. This study reinforces the Cytotoxicity Bioassay Platform's role in advancing alternative methods. The γ -H2AX HCS assay represents a major step in predictive toxicology, with the potential to reduce animal testing in compliance with 3Rs principles.

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Physicochemical properties of pesticides determine applicability of epithelial *in vitro* models to assess pharmacokinetic absorption parameters

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Toxicological effects of potentially neurotoxic pesticides observed in humans are difficult to assess in experimental animals. Physiologically relevant human *in vitro* models, in combination with Physiologically-Based pharmacokinetic (PBPK) modelling, may help to better predict toxic effects in the brain in humans. To determine target concentrations in the human brain, PBPK models can be used. The first step is to determine the absorption of pesticides via epithelial *in vitro* models (intestine and lung).

We started testing two *in vitro/ex vivo* intestinal barrier models (Caco-2 cell model, inTESTine™), and a primary bronchial epithelial cell model for their applicability to test the permeability and local toxicity of selected pesticides: chlorpyrifos, diazinon, dinoseb and rotenone. Sub-toxic concentrations, determined using the alamarBlue assay in Caco-2 cells, cultured with standard DMEM/F12 medium containing 17.5 mM glucose, were identified for chlorpyrifos ($\leq 10 \mu\text{M}$), diazinon ($\leq 100 \mu\text{M}$), dinoseb ($\leq 10 \mu\text{M}$), and rotenone ($\leq 10 \mu\text{M}$). Subsequently, these compounds were analyzed for barrier permeability and the percentage mass balance. While chlorpyrifos and dinoseb exhibit similar physicochemical properties (log P and log K_{ow}), they demonstrated a contrasting in mass balance recovery in the selected *in vitro* models, with dinoseb achieving nearly 100% recovery compared to only 10%-30% for chlorpyrifos.

In conclusion the results are of added value for the ongoing discussion to define and refine the chemical applicability domain for each *in vitro* model. The additional physicochemical property parameters we want to explore in more detail include volatility, unknown biodegradability (incomplete metabolite identification) metabolic (in) stability, biotransformation, enzymatic degradation and metabolite profiling via CYP450-mediated metabolism and biological and plastic matrix binding capacity. These parameters will be examined in relation to compounds with a good or poor mass balance, requiring the development of innovative methodologies to enhance testing accuracy.

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A 3D bioprinted and microfluidics blood-brain barrier model to study triple-negative breast cancer metastasis

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The mechanisms driving stromal invasion, neural tissue infiltration, and the transition of circulating tumor cells (CTCs) into metastatic states remain poorly understood. Existing animal models fail to capture the complex dynamics of this process and provide only limited insight into the optimal conditions for metastasis. To address this, we are developing a novel *in vitro* blood-brain barrier (BBB) model using 3D bioprinting and microfluidic technologies. This model will feature an endothelialized channel composed of endothelial cells, pericytes, and neuroprogenitor cells (NPCs), designed to mimic the interface between the BBB and neural tissue. A coaxial nozzle bioprinting system and a custom microfluidic chip with sharp curvatures are being engineered to facilitate CTC accumulation and enable controlled studies of their behavior.

To optimize the bioinks for neural and endothelial cells, we systematically evaluated various hydrogels, focusing on cell viability (assessed via Live/Dead assays and CellTiter-Glo™) and mechanical properties, including viscosity, thixotropy, and storage/loss modulus, ensuring compatibility with high-intensity microfluidic flows. GelMA-based hydrogels and decellularized brain extracellular matrix (dECM) demonstrated superior viability for neural cells (GelMA 3%, GelMA 3% + dECM), including NPCs and glioblastoma cells, over a 14-day culture period. Endothelial cells (hCMEC/D3) and pericytes exhibited high viability in GelMA 5% and GelMA 5% + Geltrex™, with endothelial cells surface-seeded and pericytes embedded within the matrix. Viability was assessed using the luminescence signal from the CellTiterGlo3D kit and Calcein AM staining.

Computational fluid dynamics (CFD) simulations using COMSOL Multiphysics™ provided critical insights into fluid behavior within the microfluidic chip. A "Z"-shaped channel design with 30° and 90° angles revealed high-pressure flow at the first curve, which may limit CTC adherence and risk bioink disruption in a 3D bioprinted model. In contrast, the second curve exhibited milder flow dynamics with low vorticity, creating favorable conditions for CTC accumulation. Additional CFD simulations were performed on "V-shaped" single-curve microchips with 90° and 30° angles to evaluate fluid distribution. These simulations indicated high shear rates and differential velocity gradients, suggesting a strong likelihood of cell accumulation in the designated geometry.

This innovative model will provide a powerful platform for studying the complex interplay between CTCs, the BBB, and neural tissue, offering unprecedented insights into the metastatic cascade of triple-negative breast cancer. By integrating advanced bioprinting, microfluidics, and computational modeling, this work lays the foundation for targeted therapeutic strategies and a deeper understanding of brain metastasis.

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Replacement of *in vivo* immunogenicity assay with *in vitro* antigenicity ELISA for pertactin and fimbriae antigens in acellular pertussis combination vaccines

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There is increasing interest to replace animal-based potency assays used routinely to test vaccines, since they are highly variable, costly, and present ethical concerns. The development of relevant *in vitro* assays offers a solution. Using the pertactin (PRN) and fimbriae (FIM) antigens as examples in DTaP-IPV (Diphtheria, Tetanus, Acellular Pertussis, and Inactivated Poliovirus) vaccine, an antigenicity ELISA was developed using monoclonal antibodies with 1) high affinity to unique PRN and FIM epitopes, 2) relevance to human immune responses, and 3) evidence of functionality. The ELISA measured consistent PRN and FIM antigenicity between vaccine lots and was more sensitive than the animal-based potency test and could more effectively detect subpotent lots, compared to the *in vivo* test. The PRN and FIM antigenicity ELISA were validated in Quality Control laboratory to demonstrate its accuracy, precision, linearity, and specificity. The product acceptance criteria were established by testing more than 30 lots of vaccine product at various ages and calculating the tolerance limits. The PRN antigenicity ELISA has been implemented as an *in vitro* replacement for the *in vivo* potency test for PRN in DTaP-IPV vaccine for release and stability monitoring. The FIM antigenicity will be implemented in the near future. This is an important milestone in the overall strategy in the vaccine industry to replace animal-based tests for component pertussis potency with *in vitro* assays.

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Mango seed butter lip balms: Safety and efficacy assessment using alternative methods based on the principle of replacement

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Concern about protecting the skin against environmental aggressors guides the creation of multifunctional cosmetics capable of preventing health problems, including sunburn, skin aging, and skin cancer. However, some areas receive less attention than others, such as the lips. Lip balms are usually made of cocoa butter; however the mango seed butter (MSB) may be a sustainable alternative raw material. MSB is rich in phenols, and vitamins and provides emollience, hydration, and antioxidant activities. Regarding the photoprotection, it is necessary the association of UV filters to cover the UVA and UVB wavelengths, being the UVA protection factor (UVA-PF) of at least one-third of the total sun protection factor (SPF). The UV filters diethylamino hydroxybenzoyl hexyl benzoate (DHHB) and tris-biphenyl triazine nano (TBPT) may be good choices, being an UVA and a broad-spectrum filter, respectively. Many countries have banned animal testing in the cosmetic industry thus alternative methods must be used in the pre-clinical phase. The 3R principles govern ethics in animal experimentation, aiming to minimize the use of animals or prioritize their well-being. The use of cell cultures corresponds to the 3R's principle of replacement because they avoid the use of animals in evaluations of toxicity and efficacy. This study aimed to develop and evaluate two different MSB lip balms: the vehicle (VLB) and the formulation containing DHHB and TBPT (FLB). To assess these formulations, they were weighed, diluted in phosphate-buffered saline and dimethyl sulphoxide, melted in a water bath at 40°C and under ultrasonication. The SPF was assessed by an *in silico* method using the software BASF sunscreen simulator, and phototoxic potential by phototoxicity test in 3T3 cells (3T3 NRU; OECD n. 432), their efficacy by using HaCaT cells to evaluate their *in vitro* antioxidant/UVA photoprotection by detection of intracellular reactive oxygen reactive species (ROS) generation using DCFH2-DA, UVB photoprotection, and anti-pollution potentials. As a result, the filters association showed a SPF estimation of 33.2, and the UVA-PF/SPF was 0.53. For the *in vitro* safety tests, none presented phototoxic potential. Regarding the *in vitro* efficacy tests, the UVA photoprotection/antioxidant activity showed significant ROS generation reduction for both lip balms at 200 µg/mL (VLB: 36,65 ± 15,89%; FLB: 46,99 ± 13,72%). For the anti-pollution potentials, this same test was performed using the particulate matter 2.5 µm (PM2.5) at 50 µg/mL, showing that both lip balms at 200 µg/mL could decrease ROS generation, with FLB being approximately twice effective as VLB (VLB: 12.41 ± 9.37%; FLB: 21.20 ± 8.07%), which can be due the presence of the UV filters. For the UVB photoprotection test, the cell viability results for FLB (80.09 ± 7.97%) were similar to the control octyl methoxycinnamate (77.35 ± 11.09%). At the same time, VLB had a lower photoprotector effect (VLB: 68,64 ± 9,05%).



Both lip balms were evaluated by using alternative methods following the replacement principle, being classified as non-toxic when in contact with the lips and not being phototoxic. They have also had potentials as: antioxidants (and UVA photoprotectors), anti-pollution formulations, and FLB as UVB photoprotector. With these results, it is possible to affirm these formulations are efficient and safe being able to proceed for clinical trials.

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Synthesis and optimization of bioinks for 3D tumor tissue models

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The probability of an oncology drug's success in clinical trials is low, partly because conventional preclinical models, such as 2D cell cultures and animal models, do not accurately replicate the tumor microenvironment or the drug response. In this context, three-dimensional (3D) bioprinted models are emerging as a promising alternative in oncology research, as they allow for the recreation of more representative tumor environments.

To address these challenges, this study aims to develop bioinks with improved biocompatibility, biodegradability, mechanical integrity, and structural stability for bioprinting colorectal tissue models. Specifically, a double crosslinking strategy was assessed, which combines ionic crosslinking with calcium chloride (CaCl₂) and covalent crosslinking with genipin at varying concentrations (0.1-0.5% w/v). Furthermore, alginate and gelatin hydrogels were formulated in different ratios and dissolved in various solvents, investigating the effect of varying genipin concentrations on cell viability and the mechanical properties of the bioinks. The hydrogels' printability, cell viability, and chemical composition were analyzed using FTIR spectroscopy, scanning electron microscopy (SEM), and cell viability assays (MTT) with Caco-2 and HUVEC cell lines.

The results indicated that the Caco-2 and HUVEC cell lines, utilized for evaluating colorectal cancer drugs, demonstrated enhanced performance on 4% gelatin, 3.5% alginate, and CaCl₂ hydrogels, as well as on hydrogels crosslinked with 0.25% w/v genipin. These findings highlight the potential application of alginate/gelatin bioinks for 3D human tissue models, emphasizing the significance of double crosslinking in optimizing bioprinted materials hydrogels.

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Promoting interdisciplinary education to facilitate the acceptance of animal-free methods

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Rising ethical concerns about animal testing for safety assessments have emerged, yet regulatory frameworks continue to heavily rely on *in vivo* studies for safety evaluations, despite its limited accuracy in predicting human toxicity. Efforts in replacing animals in safety testing focus primarily on developing alternative methods. However, it is equally important to validate these methods, integrate them into regulatory frameworks, ensure public acceptance and increase awareness among young scientists via education and training modules. Just like animal testing, animal-free safety testing carries inherent uncertainties that hinder its broader adoption. To overcome this, a paradigm shift is needed to embrace these uncertainties. This transition requires collaboration with fields like social sciences, law, psychology, and economics, as they can offer valuable insights into how these changes can be achieved. Moreover, it is vital to identify and collaborate with stakeholders with a variety in backgrounds responsible for implementing alternative methods.

Collaboration across disciplines can be challenging. While differences in terminology between disciplines are commonly recognized, more fundamental differences often go unnoticed. These include varying perspectives on what constitutes facts, how knowledge is acquired and what qualifies as acceptable evidence for research or regulatory purposes. Additionally, hierarchical distinctions between disciplines may exist. Recognizing and addressing these differences is essential for successful interdisciplinary collaboration. To navigate these challenges, specialized training in interdisciplinary skills is crucial.

A straightforward exercise can help students and researchers recognize the strengths and barriers of their own disciplines. This so-called "disciplinary humility" paves the way for collaboration across different fields and is considered the first step for developing interdisciplinary skills in researchers. The exercise was also used in a consortium meeting for the Dutch Virtual Human Platform for Safety (VHP4Safety) project, which aims to develop animal-free testing strategies for safety assessment of chemicals: strategies that can be accepted and implemented by regulators. Discussions between researchers, regulators and other stakeholders revealed limitations in knowledge of their respective disciplines as for instance uncertainties of experimental data or understanding human behavior for risk communication and led to identification of educational needs among various project members. This resulted in development of tailored workshops for researchers within the consortium with specific backgrounds to enhance their knowledge in areas they are unfamiliar with. Examples include a workshop on principles of toxicol-



ogy for researchers from social sciences and a workshop for regulators and other stakeholders on mission-specific innovation systems. These tailored educational offerings will improve mutual understanding and support the transition to the acceptance of animal-free alternative methods for predicting human toxicity. The approach can be easily translated to other projects that aim to increase development and acceptance of animal-free methods.

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Evaluation of the applicability of alternative *in vitro* methods in Brazil: A literature review

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Animal experimentation has been widely used to assess the safety of cosmetic, pharmaceutical, and personal hygiene products. However, ethical and scientific concerns drive the development of alternative methods, such as *in vitro* tests, which utilize cell cultures and reconstructed human tissues. These methods offer greater accuracy, lower costs, and eliminate animal suffering.

This study aimed to conduct an integrative review of Brazilian literature on the application of validated *in vitro* methods, analyzing articles

published between 2019 and 2024, with a focus on methods recognized by the National Council for the Control of Animal Experimentation (CONCEA) and based on the guidelines of the Organization for Economic Cooperation and Development (OECD). To this end, research was conducted using the following databases: BVS, CAPES CAFE, and Google Scholar, with the OECD method codes as descriptors.

The results indicate that, among the 28 recognized methods, only 11 were addressed in the analyzed articles, highlighting both growth in their use and challenges in their implementation. Skin and eye irritation tests were the most frequently cited, particularly “OECD TG 492 – Reconstructed Human Corneal Epithelium” and “OECD TG 439 – *In vitro* Skin Irritation Test,” which are widely used in the assessment of substance toxicity. For phototoxicity and genotoxicity, “OECD TG 432 – *In vitro* 3T3 NRU Phototoxicity Test” and “OECD TG 471 – Bacterial Reverse Mutation Test” demonstrated results equivalent to those of animal models, with reduced time and costs. Methods such as the “Monocyte Activation Test (MAT),” “OECD TG 442D – *In vitro* Skin Sensitization,” and “OECD TG 442E – *In vitro* Skin Sensitization: Assays Addressing Dendritic Cell Activation” were effective in identifying allergens and ensuring the safety of injectable products.

Despite advancements, challenges still need to be overcome. The lack of standardization in laboratory protocols, the need for investment in infrastructure, and professional training hinder the full implementation of these methods in Brazil. Regulatory acceptance and the adaptation of industrial processes are barriers that require greater attention to ensure the effective replacement of animal testing. Brazilian legislation has advanced in promoting these methods, particularly with CONCEA’s Normative Resolution No. 58 (2023), which mandates the use of alternative methods for cosmetic testing whenever available. This regulatory milestone represents significant progress but requires rigorous enforcement to ensure compliance.

Replacing animal testing with *in vitro* methods represents both an ethical and scientific advancement, ensuring greater accuracy in experimental results. The global trend indicates an increasing adoption of these methodologies, making continuous investment essential to consolidate their application in our country. This study reinforces the importance of alternative methods and the need for coordinated efforts to enable their broad and effective implementation.

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IPAM, the Italian platform on alternative methods

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National Platforms for alternative methods were created about twenty years ago under the umbrella of the European Consensus Platform for alternatives (ecopa; <https://ecopa.eu>) with the aim to promote and inform about alternative methods to speed up their regulatory acceptance. To achieve these goals effectively and incisively, collaboration between the following four areas was considered as a priority: Research – Industry – Governmental Institutions – Associations for animal protection.

The Italian Platform on Alternative Methods (IPAM), in full compliance with this approach, pursues at: i) promoting research and information on alternative methods in animal experimentation in Italy, ii) building synergies to accelerate development and acceptance of alternatives methods in basic, applied, and regulatory research. Within this framework, IPAM regularly organizes national and international events and meetings [1,2], including the webinar's cycle "3Days-for-3Rs" [3,4] aimed at different stakeholders (students, researchers, general public). Among some of the most relevant initiatives, the exhibition "Science & Consciousness, a journey inside the 3Rs" a didactic itinerary on alternatives in animal experimentation which was hosted by several Italian universities and research institutes, and the IPAM-ecopa

symposium 2019 on "Non Animal Methodologies (NAMs): research, testing, assessment and applications", co-organized with ecopa [5].

Every two years, alternatively, an IPAM award is assigned to either a young researcher author of a paper and/or a thesis degree relevant to the application of 3Rs Principle in pharmacological research (the IPAM-Farmindustria award, since 2017) or to a graduate/doctoral thesis relevant to New Alternative Methods (NAMs) and the 3Rs principle (IPAM thesis award, since 2020).

IPAM also actively dialogues with national and international regulatory bodies and its members frequently share their expertise in training events organized by university, industries, and public entities.

The IPAM's website (<https://www.ipamitalia.org>) and Facebook social (www.facebook.com/IPAMITALIA) represent an important point of reference of information, updates, and discussion for anyone who is interested on the 3R Principle, alternative methods and their applications in science.

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Microgut: Biology-inspired intestine epithelial model using microfluidic-based gelatin methacrylate microgel to create reverse-polarity HT29 spheroids

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The *in vitro* model for the human gut is a significant tool for predicting drug absorption and toxicity, as well as for understanding infectious diseases due to interactions between pathogens and hosts. The intestinal microenvironment has several biochemical and morphological features aspects. The villi curvature generates a signal that guides the cells' polarization, migration, and differentiation, a phenomenon called curvotaxis. In this study, we replicate the tops of intestinal villi (convex surfaces) using gelatin methacrylate (GelMa) microgels. GelMa microgels serve as valuable tools for immobilizing cells for cell delivery in injectable therapy. However, it remains unclear how the hydrogels and the curvature of the microparticles influence the morphology and physiology of the cells. Herein we present our work previously published (Bianchi et al., 2025), which we created the microgut: reverse-polarity spheroids using GelMa microgels produced by droplet-based microfluidics. Microgels were used at varying concentrations (7 and 10% w/v) and stiffness levels, with a Young's modulus of approximately 0.01 kPa, produced through flow-focusing droplet microfluidics optimizing the flow rate ratio of hydrophobic and hydrophilic phases to obtain microgels roughly 80 µm in size. Thus, we cultured HT29 cells around the GelMa microgels to mimic intestinal mucus-producing cells (such as Goblet cells) and examine curvotaxis in relation to cell polarization for creating reverse-polarity spheroids. The HT29 cells on GelMa hydrogels maintained a high viability of approximately 95% after five days of culture. Immunostaining revealed that the cells polarized in response to the curvature of the microgel, with cell nuclei oriented toward the gel and a high expression of F-actin on the apical side. Additionally, the HT29 cells cultured on the GelMa7 microgel exhibited higher expression of tight junctions compared to those on the GelMa10 microgels. We refer to this cell-laden microstructure, characterized by reverse-polarity spheroids formed by cell growth around microgels, as the microgut. The microgut model provides improved access to the apical side membrane, better mimicking the epithelial layer, exposing access to the apical membrane. Its potential applications include drug absorption, toxicity assessments, and infectious disease research.

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Advancing organ-on-a-chip technology: Global trends, regulatory perspectives, and implications for preclinical testing

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Introduction: The use of animals as preclinical models for drug testing has been increasingly questioned due to ethical concerns and their limited predictive capacity for human efficacy and safety. Despite major investments in analytical tools and standardization, only a small fraction of Phase 1 drug candidates receives FDA approval. Clinical trial failures are often linked to adverse reactions and inefficacy, underscoring the need for alternative preclinical methods with greater reliability, lower costs, and broader accessibility. In this context, organ-on-a-chip (OoC) technology has emerged as a promising alternative. OoCs are advanced cell culture platforms that replicate human organ functions under controlled fluid dynamics. Their potential to surpass animal models' limitations has driven a surge in scientific publications and patents, making this technology central to discussions in industry, academia, and regulatory agencies.

Objective: To characterize the global landscape of the development, adoption, and regulatory acceptance of OoC devices as alternatives to animal models.

Methods: A comprehensive document analysis was conducted. Systematic searches were performed in the CAPES journal database, the PatentScope platform (World Intellectual Property Organization – WIPO), regulatory agency websites, corporate sites of OoC manufacturers, scientific societies, and the Tracking System for Alternative Methods Review (TSAR) tool.

Results: OoC development has grown significantly. Between 2015 and 2024, approximately 93,808 patents were filed worldwide, with the U.S. leading (65,514 applications). In Brazil, six patents were identified, five from national research. While OoCs are not yet included in official regulatory guidelines, collaborative efforts among governments, industries, and universities are accelerating alternative methods to animal testing. In Europe, initiatives like ORCHID and EUROoCS have been established, while in the U.S., programs such as Tissue Chip for Drug Screening (National Center for Advancing Translational Sciences) and Innovative Science and Technology Approaches for New Drugs (FDA) address standardization and validation. The FDA Modernization Act 2.0, which reinforces the validation of New Approach Methodologies (NAMs), including *in silico* models, creating opportunities for regulatory integration, was a key regulatory milestone globally. In Brazil, Anvisa has engaged in scientific outreach on this technology but states that OoCs are not yet applicable for assessing the biological safety of medical devices.

Conclusions: OoCs hold great potential to overcome animal model limitations in preclinical testing. The increasing number of patents and international initiatives demonstrates growing recognition of its poten-



tial to enhance drug testing reliability while reducing reliance on animal models. Brazil has already made significant strides in this field, with national research contributing to patent filings and Anvisa engaging in scientific discussions. Continued investment in reproducibility assessments, standardization, and alignment with regulatory requirements will be key to accelerating the transition from experimental models to widely accepted alternatives in preclinical testing.

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Optimizing Wnt activation in fetal calf serum (FCS)-free organoid expansion media

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Organoid technology can revolutionize biomedical research by increasing the translational value of experimental results while at the same time reducing the need for experimental animal use. However, in most cases the organoid culture workflow relies on expansion media that contain fetal calf serum (FCS). The production of FCS causes animal suffering, and the use of it is hampered by factors that negatively impact the reproducibility (such as the large inter-batch variation and the undefined composition of FCS), relevance (such as the induction of a non-physiological cellular phenotype), as well as the clinical translatability (such as the potential to cause xeno-immunization or to contain xenogenic pathogens). There is thus a strong impetus to find animal-free alternatives to the use of FCS.

Most contemporary expansion media for organoid culture are not FCS-free. This is mainly attributable to the use of FCS for the recombinant production of the growth factor Wnt3A. Wnt3A-conditioned medium is added to expansion media to induce Wnt signaling, which is necessary for organoid proliferation. In turn, FCS is pivotal to stabilize and solubilize the Wnt3A protein, and not perse for the survival, adhesion or proliferation of cells. We will summarize alternative methods to induce Wnt signaling in organoid expansion media, encompassing the use of soluble Wnt mimetics, the use of carriers, and the use of small molecule inhibitors. Alternative Wnt activation approaches for different experimental goals will be presented and discussed.

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The environmental impact of the production of animal-derived laboratory products

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Following the societal desire to decrease our reliance on experimental animals, *in vitro* research has established itself as a valuable alternative to replace and reduce animal experiments. However, *in vitro* workflows often still require animal-derived products (ADP). Especially basement-membrane extract (BME – brand names Matrigel®, Geltrex™, Cultrex™), fetal calf serum (FCS) and animal-produced antibodies (APAs) are widely used. The use of ADP goes accompanied by ethical and scientific concerns, and account for substantial numbers of invisible animal use and associated suffering.

Environmental impact assessments of cow farming and experimental animal facilities, as well as the results from a pilot study, suggest that the production processes of BME, FCS and APA are far from sustainable and negatively impact the environment. Nevertheless, systematic analyses of the environmental impact of the production processes are lacking, which represents a major knowledge gap.

We will present our plans to conduct Life Cycle Assessments (LCAs) for BME, FCS and APA. All LCAs consist of a goal and scope definition, an inventory analysis, an impact assessment, and an interpretation step. Information from literature and collaborations with animal facilities and companies will be used to accomplish this. The combination of data from several resources and the expertise of our interdisciplinary team will help to minimize data gaps and uncertainties.

The results will identify ways to reduce negative impacts of ADP production and will enable comparison with the impact of other production processes. Thereby they will provide tools to reduce environmental consequences of biomedical research. Furthermore, they will facilitate the inclusion of sustainability aspects to the scientific and societal discussion about animal use in science.

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Deriving a point of departure for assessing skin sensitization risk of chemicals using the SENS-IS assay

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Background and purpose: Skin sensitization risk assessment is essential in toxicology, especially given the push toward non-animal testing methods. The SENS-IS assay, an *in vitro* test, offers a promising alternative for identifying and classifying the skin sensitization potential of chemicals. Previously, using a panel of 139 reference chemicals from the OECD's list, the SENS-IS assay demonstrated robust accuracy – 95% in identifying sensitization hazards and 86% in classifying sensitization potency according to UN-GHS criteria. However, existing *in vitro* methods are limited by an inability to define No Expected Sensitization Induction Level (NESIL) values between tested concentrations. This study aims to refine the SENS-IS assay's ability to determine a continuous point of departure (PoD) for sensitization risk, potentially improving its precision and relevance for regulatory use.

Methods: We compiled a comprehensive database of 139 reference chemicals, each tested across multiple concentrations. The chemicals were assessed for expression of the 61 gene biomarkers utilized in the SENS-IS assay. Advanced artificial intelligence (AI) and machine learning techniques were then applied to analyze gene expression patterns and concentration data, allowing us to derive continuous PoD values. This AI-enhanced model was then compared to traditional *in vivo* data from LLNA EC3 values and human No-Observed Effect Level (NOEL) data to assess consistency and predictive accuracy.

Results: The application of AI-driven analysis successfully allowed the derivation of continuous PoD values for each tested chemical. Our findings revealed a strong correlation between the SENS-IS-derived PoD and traditional LLNA EC3 and NOEL values, confirming the assay's predictive accuracy. This correlation suggests that SENS-IS, enhanced with AI, could reliably serve as an *in vitro* alternative for assessing chemical sensitization risk and provide refined NESIL values across a broader range of concentrations, supporting more nuanced risk classifications.

Conclusions: Integrating AI analysis with SENS-IS assay data represents a significant advancement in skin sensitization risk assessment, achieving high accuracy in hazard identification and potency classification. This approach allows for the derivation of reliable PoD values across various concentrations, offering an improved tool for regulatory toxicology. It thus contributes to the movement toward animal-free testing and presents a robust, ethical alternative for sensitization testing, with the potential to replace traditional animal-based methods in regulatory settings.

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Educating for change: The role of students in advancing laboratory animal-free cell culture research

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The transition toward laboratory animal-free research requires not only technological advancements but also a new generation of scientists critically trained in non-animal-based models. In alignment with the 3Rs principle, the 3Rs Centre Utrecht and Utrecht University of Applied Sciences have established innovative educational research programs that integrate challenge-based learning with experimental cell culture projects. These initiatives aim to replace animal-derived components in cell culture media, with a strong focus on alternatives to fetal calf serum (FCS), while providing students with an authentic research environment.

FCS, widely used in cell culture, presents ethical concerns and experimental limitations due to its undefined composition and batch variability. In 2024, third-year bachelor students enrolled in the Innovative Laboratory Animal-Free Methods course evaluated serum-free alternatives for cell types widely used in *in vitro* models, including muscle development (C2C12), cancer (DAOY), gene/protein expression studies (HEK293), and immune system research (THP-1). Tested alternatives included commercially available serum-free media (Proliferum M and Panexin Basic) and a defined non-commercial medium [1,2].

Preliminary results demonstrated that Proliferum M supported the proliferation of C2C12 cells; however, horse serum remained necessary for differentiation. DAOY and THP-1 cells exhibited suboptimal growth in Panexin Basic, whereas the defined medium facilitated a 75% reduction in FCS for HEK293T and THP-1 cells without compromising cellular morphology. Furthermore, both cell lines retained functional performance, as evidenced by unaltered transfection efficiencies in HEK293T cells and PMA-induced differentiation into macrophages in THP-1 cells. Notably, both cell lines remained viable following cryopreservation and subsequent thawing under serum-free conditions.

Supported by the FCS-free database (<https://fcs-free.sites.uu.nl/>) and collaborations within Utrecht Science Park, this project highlights the feasibility of reducing serum dependency in routine cell culture. More importantly, it underscores the essential role of integrating education, research, and industry collaboration in driving the adoption of animal-free methodologies. By training future scientists in innovative non-animal research techniques, we contribute to global 3Rs efforts while enhancing experimental reproducibility and ethical research practices.

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Bridging the gap: A helpdesk to connect NAMs experts with stakeholders

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New Approach Methodologies (NAMs) have immense potential for real-life applications such as disease modelling, drug development and personalized medicine. As much as the field is driven by technical advances that increase the applicability of NAMs for human physiology, the potential of NAMs to reduce or replace experimental animal use remains relatively underappreciated.

One of the challenges is to effectively translate the knowledge generated in research labs to other organizations. Researchers that are active in the field of NAMs hold highly specialized knowledge and expertise, whereas stakeholders across academia, industry and regulatory sectors often face difficulty assessing such expertise. An exploratory study conducted by the 3Rs Centre Utrecht (3RCU), of which we will present the results, revealed a substantial demand for accessible expertise in animal-free innovations. Especially stakeholders such as animal welfare bodies and ethical committees are in need of such expertise.

To address this, the 3RCU is exploring ways to connect NAMs experts with stakeholders seeking guidance on specific questions. For this we are currently testing how a helpdesk functions that is linked to a database of NAMs experts. Questions can be related to issues ranging from technical information on the performance and/or validation of NAMs, to the ability of NAMs to reduce or replace the use of experimental animals for certain research questions. The helpdesk offers an organized, collaborative environment that supports both experienced consultants and those new to such roles, enabling them to engage directly with stakeholders who need their insights. The initiative is supported by the Dutch government program "Innovation without the use of animals" and is performed in collaboration with the European consortium COST Action IMPROVE.

This initiative provides the opportunity for NAMs researchers to increase their impact by bridging the gap between research and application. We therefore invite NAMs researchers to join this initiative, leveraging their expertise to drive real-world change. By joining the database, researchers can share their expertise with a broader audience, contribute to reducing the reliance on animal models, and help shape the future of biomedical science.

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Refining animal experiments: An extensive update of the humane endpoints website

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A humane endpoint is defined as the earliest indicator in an animal experiment of (potential) pain and/or distress that can be used to avoid or limit pain and/or distress by taking actions. Taking actions does not necessarily mean the humane killing of the animal but also includes conducting interventions to alleviate the stress- or painful experimental procedure or providing analgesics.

The widely used, publicly accessible Humane Endpoints website (<https://www.humane-endpoints.info>) has been developed to facilitate professionals who work with laboratory animals to define, recognize and implement humane endpoints in their work. The website and associated e-learning is not only suitable for education and training purposes but also serves as a reference tool. The site currently contains extensive information on mice, rats and zebrafish.

The Humane Endpoints website is part of the 3Rs Research Tools Program, which is managed and hosted by the 3Rs Centre Utrecht. Currently, we are conducting a major update of the website, and we will present our plans here. The aim is to improve the user experience by visual updates. Moreover, we are updating the content and complementing it with additional information.

In addition, we aim to enhance the international embedding of the Humane Endpoints website by seeking feedback from international experts on laboratory animal behavior. This to ensure that the website remains the go-to tool for professionals in the future to improve their knowledge on humane endpoints and animal welfare, to refine their experiments and to improve the scientific quality of their research.

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New approach methodologies advancing identification of potential endocrine disrupting chemicals

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In April 2023 a Commission Delegated Regulation (EU) 2023/707 entered into force. It introduces new hazard classes and criteria for the classification, labelling, and packaging of substances and mixtures (CLP), specifically focusing on endocrine disruptors (ED) and persistent, bioaccumulative, toxic and mobile chemicals (PBT and PMT). According to the definitions provided by World Health Organization (WHO) and European Commission, a chemical can be identified as an ED if it meets specific criteria. These include an endocrine mode of action, a demonstrated adverse effect in an intact organism, and a biologically plausible link between the adverse effect and the endocrine mode of action.

Current testing for regulatory purposes heavily relies on validated methods which frequently involve extensive use of live animals. Identifying a chemical as an ED necessitates complex testing strategies that are hampered by the lack of validated methods, unexplored endocrine pathways and uncharacterized long term adverse effects. Therefore, the testing strategies for the assessment of ED should integrate different approaches and data. New Approach Methodologies (NAMs) applied in combination have the potential of delivering relevant information on chemical hazard and risk assessment without the use of animals, thus, relying on *in silico*, *in chemico*, *in vitro*, and *ex vivo* studies. Considering the immediate necessity to identify potential ED substances while minimizing animal testing, we present an *in silico* NAM-based workflow for the indication of potential Estrogen, Androgen and Thyroid hormones mediated effects. The workflow utilizes validated methods and integrates various approaches with existing data including (Q)SAR models, identification of structural analogues, data on structural analogues, existing data from traditional testing and endocrine activity related *in vitro* assay data from ToxCast21 program. The workflow offers a transparent, reproducible and robust solution to inform decision-making processes in relation to the EAT modalities. It can be utilized as a stand-alone tool for screening and priority setting, for example to streamline testing in alignment with the OECD conceptual framework for testing and assessment of endocrine disruptors. Lines of evidence delivered by the workflow can inform a weight-of-evidence assessment regarding the applicability of the CLP criteria for assigning ED classifications. The workflow is designed for scalability, allowing application to large batches of substances, and has the potential for automation, thereby enhancing the efficiency and effectiveness of chemical assessments.

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Investigating editorial perceptions on *in vitro* methods to assess physiological relevance

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The mission of the 3Rs Centre Utrecht (3RCU) is to stimulate the development, acceptance and implementation of methods that can Replace, Reduce or Refine animal experiments. To do so, we try to form a bridge between researchers working with New Approach Methods (NAMs), and researchers that are using experimental animals.

In close collaboration with the EU COST Action IMPROVE, we are investigating how biomedical researchers, and in particular journal editors, perceive the value of *in vitro* and *in vivo* data when assessing physiological relevance. A central dilemma is whether interspecies extrapolation of *in vivo* data is more trustworthy than intermethod (*in vitro-in vivo*) extrapolation within the same species. To further explore this topic (also known as animal bias), we developed a survey which has been widely distributed amongst editors of scientific journals. The survey contains questions about handling referee's requests for additional *in vivo* data, about journal policies concerning *in vivo* data, and about editorial knowledge of, and attitude towards, NAMs. Scientific journals were selected from the 10 fields of biomedical research with the highest prevalence of experimental animal use.

We will here present the survey along with some preliminary results. These results will be integrated in a letter about the perceived value of NAMs that is to be submitted to a relevant biomedical journal.

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Assessment of development and reproductive toxicology (DART) of benznidazole: An *in vitro* approach

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Current options for Chagas disease treatment are limited; one of the drugs commonly used is benznidazole (BZ). Due to the lack of information, etiological treatment is not recommended in pregnant women. Using an integrated *in vitro* approach, the results can provide comprehensive information on the BZ potential effects on gametes and embryonic development, decreasing the number of animal studies and providing a more detailed toxicological profile. The aim of this study was to evaluate the *in vitro* effect of BZ on bovine sperm viability and motility, bovine oocytes *in vitro* maturation (IVM), and *in vitro* fertilization (IVF). To evaluate the toxicity on male gametes, bovine spermatozoa were exposed to increasing concentrations of BZ for 1 hour studying viability with eosin-nigrosin vital stain along with motility and velocity parameters assessed by computer-assisted sperm analysis. To assess the effect during oocyte IVM, cumulus-oocyte complexes were obtained from ovaries of slaughter cows, selected and incubated in IVM medium with increasing BZ concentrations. After 22 h, oocyte viability was evaluated with Trypan blue and nuclear maturation with Hoechst stain. To evaluate the effect during IVF, bovine gametes were co-incubated with increasing concentrations of BZ. After 20 h, penetration was evaluated with Hoechst stain to determine the concentration that reduced the IVF by 50% compared to the untreated control. Sperm analysis showed that none of the parameters was significantly modified compared with the untreated control (one-way ANOVA). BZ did not affect oocyte viability. The IC₅₀ for IVM was 222.4 μ M which is above the limit previously established in the validation of the assay of the potentially toxic concentration for oocytes (≤ 50 μ M). This would indicate that BZ does not significantly affect the bovine IVM of oocytes. The preliminary calculation of IC₅₀ for IVF was 318.5 μ M. This initial battery of *in vitro* models is part of developing a biotechnological platform for DART testing, which can be applied in several potential toxic substances, promoting the technological cooperation between academia and industry partners within a 3Rs approach.

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Evaluation of a fluorescent gelatin proteolytic assay of *Bothrops asper* venom as an alternative of lethality neutralization tests of said venom

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Every year, approximately 2.3 million people worldwide suffer from snakebite envenoming, a condition where venomous snakes inject their venom into humans. The only therapeutically validated treatment is the intravenous administration of antivenom, a biotechnological drug derived from hyperimmune equine plasma. Without timely antivenom administration, said envenoming can be deadly; meanwhile, survivors often face long-term sequelae that impact their quality of life such as reduced mobility and amputation. Additionally, the psychological toll of these conditions remains underexplored. Moreover, to assess antivenom efficacy, manufacturers routinely conduct *in vivo* lethality neutralization tests, which evaluate survival or mortality rates in mice injected with venom-antivenom mixtures. Although these tests are recognized by the World Health Organization (WHO) as reliable indicators of efficacy, they raise ethical concerns due to their use of laboratory animals. Reducing the use of laboratory animals in the antivenom production process is essential. Therefore, many studies have worked to develop *in vitro* techniques that more accurately reflect an antivenom's neutralizing capacity. However, lethality tests continue to be the "gold standard" for demonstrating preclinical antivenom efficacy.

Viperidae snakes are responsible for a significant number of envenoming cases worldwide. The complications of Viperidae bites are linked to hemotoxic and hemorrhagic events, caused by metalloproteinases (MPs) in the venom. These toxins can mimic human coagulation factors and degrade tissue proteins, leading to coagulation disorders and systemic hemorrhage, which contribute to the venom's lethality. At the Instituto Clodomiro Picado (ICP), we produce PoliVal-ICP® antivenom to treat snakebites from Viperidae species in Central America, the Caribbean, and several South American countries. We have previously shown that lethality neutralization values – routinely generated by the ICP Quality Control Laboratory – correlate with an in-house designed *in vitro* procoagulation neutralization test for *Bothrops asper* venom. Furthermore, we have demonstrated that a fluorescent gelatin proteolytic assay can effectively assess the activity of *B. asper* venom and its neutralization by PoliVal-ICP®.

The objective of this project is to establish the fluorescent gelatin assay as a reliable test to assess the neutralization of *B. asper* venom's proteolytic activity at different stages of the antivenom production process. We also aim to evaluate its correlation with the neutralization of the venom's lethality and procoagulant activity. Currently, we are evaluating the pro-



teolytic activity neutralization capacity in hyperimmune serum samples from horses immunized with venom mixtures for PoliVal-ICP® production, and samples from different stages of the PoliVal-ICP® antivenom production process. Preliminary results suggest a correlation between the neutralization of proteolytic activity on fluorescent gelatin and the neutralization of both lethal and procoagulant activities of *B. asper* venom.

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Hut handling as an effective and practical alternative to cupping and tunnels for refined mouse handling

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Virtually all laboratory mice are handled during experimental procedures and background husbandry. Mice are commonly tail-handled, despite evidence that this is aversive. Established alternatives include cupping mice with open-gloved hands or with tunnels; both alternatives are associated with improved welfare outcomes, including reduced anxiety, increased voluntary interaction with the handler, and improved ease of handling. However, tail handling persists due to various real and perceived barriers to adopting refined methods, including the cost of purchasing tunnels, concerns about in-

creased handling time, and the perception that common procedures will be more difficult to perform. The practicality of refined handling may be improved by using handling objects already present in facilities, such as upturned mouse huts. Our aim was to compare hut handling with the established alternatives of cupping and tunnel handling. Since both tunnels and huts may be provided as a shelter within the cage, a second aim was to assess the effects of object familiarity (i.e., always present in the home cage vs. a clean, novel handling object). Outcomes assessed were voluntary interaction with the handler and time spent in the open arms of an elevated plus-maze (EPM), a validated behavioural test of anxiety in rodents. Mice ($n = 51$) were randomly assigned to a handling method: cupping, tunnel, or hut. Cages ($n = 14$) were randomly assigned to contain a shelter (tunnel or hut). Mice housed with the shelter type assigned to them were handled using this familiar shelter, and those housed with a different shelter were handled using a clean, novel shelter. Mice underwent 9 days of brief handling sessions and voluntary interaction tests were conducted on days 1, 5, and 9. On day 10, mice were tested in the EPM. We found that voluntary interaction with the handler varied with handling object: hut-handled mice spent the most time interacting with the handler, followed by tunnel-handled and cupped mice (41.7 ± 1.5 s, 36.1 ± 1.4 s, and 33.0 ± 1.5 s, respectively). Familiar objects improved interaction at the outset, but this difference was no longer evident by day 5. We found no effect of handling object or object familiarity on time spent in the open arms of the EPM. These results suggest that handling mice with a familiar home cage shelter is preferable to an unfamiliar one, but habituation to handling with a novel shelter is achievable after multiple handling sessions. Hut handling is an effective and practical alternative to cupping and tunnel handling, particularly in facilities that already have access to huts.

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The case for justify: Empowering lab workers and advancing ethical science

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Recent scholarship and journalism have revealed that animal research operates within a culture of secrecy, fear, and ethical conflict. Lab workers – often drawn to the field out of a desire to care for animals or improve human health – experience moral injury, burnout, and psychological distress.

Researchers and caregivers have reported that the industry conditions them to view any discomfort with their responsibilities as weakness or a rejection of science itself. Expressing concerns about the ethical implications of their work can lead to professional and social repercussions. Meanwhile, the broader public remains largely unaware of these hidden struggles, and the lack of transparency hinders progress toward ethical, human-relevant research alternatives.

Conceived in 2020, Justify began as an educational platform, shedding light on the hidden toll of animal research on both lab workers and animals. The overwhelming response – particularly from those within the industry – made it clear that a deeper level of support was needed.

In 2024, we began the process of establishing Justify as a nonprofit, committed to providing resources, advocacy, and community to animal research personnel while advancing ethical, human-relevant science. Founded by former lab workers who experienced firsthand the comfort of confiding in each other about their shared background, Justify is a groundbreaking organization that provides a safe and supportive space for those grappling with the emotional burden of animal research, empowering them to share their experiences and advocate for change.

Justify is the only organization of its kind working to gather, advocate for, and heal individuals from the animal experimentation industry – led entirely by individuals with firsthand experience working in the industry.

Justify operates based on these key assumptions: 1) Transparency leads to change (increased visibility into the emotional and ethical toll of animal research will shift public perception and policy); 2) Empowered lab workers drive progress (by supporting lab workers in speaking out and transitioning to ethical research, we create momentum for systemic change); 3) Dialogue over division (bridging the gap between researchers and advocates fosters collaboration toward humane scientific progress).

Open dialogue about the ethics of animal research and its psychological impact on lab workers is essential. Silencing these discussions – whether by creating a culture of fear or through more direct interventions – contradicts the core mission of scientific research: advancing knowledge and improving both human and animal wellbeing.

Through education, community, and action, we address the normalization of animal experimentation as a profession and promote innovative, animal-free research methods. Justify is catalyzing a movement – one where lab workers are heard, science prioritizes compassion, and a more just future for humans and animals becomes possible.

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Functional mapping of neurodevelopmental disease pathways and nuclear hormone receptor signaling to key neurodevelopmental processes represented in the developmental neurotoxicity *in vitro* testing battery

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Developmental neurotoxicity (DNT) testing is critical to safeguarding healthy brain development, yet current regulatory approaches remain inadequate. Traditional *in vivo* studies are resource-intensive, suffer from species differences limiting human relevance, and lack mechanistic insight. Consequently, the vast majority of environmental chemicals including endocrine-disrupting chemicals (EDCs) remain untested for DNT hazard. To address this gap, human-based new approach methodologies (NAMs) have been developed, including the DNT *in vitro* battery (IVB). However, for NAMs to gain regulatory acceptance, a deeper understanding of their biological relevance and pathophysiological applicability domain is required.

The Neurosphere Assay, an integral component of the DNT IVB, models key neurodevelopmental processes (KNDPs) using primary human neural progenitor cells (hNPCs). In this study, we investigated how signaling pathways and nuclear hormone receptors (HRs) regulate KNDPs assessed in the Neurosphere Assay – such as NPC proliferation, radial glia, neuronal, and oligodendrocyte migration, neurite outgrowth, and neuronal and oligodendrocyte differentiation. Using a panel of 31 disease pathway modulators and 14 HR agonists and 12 antagonists, we assessed their concentration-dependent effects on the KNDP regulation. We further analyzed the expression of HRs and key pathway-related proteins in proliferating and differentiating hNPCs using microarrays and compared them to corresponding expression data from the human fetal cortex available in the BrainSpan database. Finally, we mapped the observed signaling pathway-mediated regulation of KNDPs to human neurodevelopmental disorders to further increase confidence in DNT IVB assays for human health protection and hence their regulatory use.

Our results demonstrate that the Neurosphere Assay has the potential to effectively detect interference from environmental chemicals, including endocrine disruptors, in most tested signaling pathways (16 out of 18) and HRs (12 out of 14), except signal transducer and activator of transcription 3 (STAT3), tropomyosin receptor kinase B (TrkB), and androgen receptor (AR), and estrogen receptor (ER), which fall outside the assay's applicability domain. Crosstalk between HRs and the involvement of developmental signaling pathways in the HR KNDP regulation, such as Notch and Wnt, further underscores the intricate regulatory interactions governing neurodevelopment. Among the assessed KNDPs, oligodendro-



cyte differentiation emerged as the most strongly regulated, highlighting the complex signaling required for oligodendrocyte development and identifying it as the most sensitive functional endpoint for detecting DNT-relevant effects. Based on these findings, we propose the development of human-based NAMs designed to identify EDCs that disrupt critical KNDPs by interfering with key hormonal signaling pathways.

By mapping the functional impact of neurodevelopmental signaling on KNDPs, we increased the regulatory confidence in the Neurosphere Assay by enhancing its mechanistic interpretation, characterizing its biological applicability domain, and relevance to human physiology and diseases. Strengthening the scientific foundation of *in vitro* approaches is essential for advancing their regulatory acceptance and ultimately improving human-relevant DNT risk assessment.

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A path forward advancing micro-physiological systems

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Microphysiological systems (MPS), including organ-on-chip platforms and organoid models, represent a transformative approach to human-relevant *in vitro* modeling. These technologies are revolutionizing drug de-

velopment and enabling personalized medicine while reducing animal testing. Despite significant advances, several critical challenges must be addressed to realize their full potential. Standardization remains a fundamental challenge, with inconsistencies in terminology, protocols, quality standards, and data reporting practices creating barriers to reproducibility. Validation and benchmarking also present significant hurdles, particularly in identifying appropriate reference compounds and establishing acceptance criteria for regulatory purposes. Limited data sharing due to proprietary concerns, absence of standardized formats, and regulatory uncertainty further impede progress.

The integration of artificial Intelligence (AI) offers promising solutions, including AI-driven digital MPS emulations that enable prediction of treatment responses, optimization of system parameters, and real-time monitoring of multi-dimensional data streams. Additionally, machine learning approaches can automate analysis, enhance predictive modeling, and support validation processes.

To overcome these challenges, we propose establishing global standards through international consortia, enhancing validation frameworks via specialized validation centers, fostering pre-competitive collaboration through secure data-sharing platforms, and advancing regulatory integration through clear guidance documents and regulatory science initiatives.

Successful implementation requires coordinated efforts across academia, industry, regulatory agencies, and technology suppliers. The path forward includes short-term standardization efforts, medium-term validation studies and data-sharing initiatives, and long-term transformation of drug development paradigms to fundamentally improve human health outcomes while significantly reducing animal testing.

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Animal-free assays for endocrine disruption – From science to regulatory acceptance (AFARA): *In vitro* test model development

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Metabolism disrupting chemicals (MDCs) are substances capable of interfering with processes in organisms involved in metabolism and energy homeostasis. These chemicals are present in various industrial products, food additives, and consumer products and can disrupt metabolic pathways via the endocrine system. A range of health issues, including obesity, diabetes, and metabolic dysfunction-associated steatotic liver disease (MASLD) have been associated with MDC exposure. As society is dealing with ubiquitous presence of these chemicals, understanding their impact on human health is warranted. However, there is a lack of standardized test methods. Within current European projects models are developed to accurately predict the effects of (suspected) MDCs in humans, using new approach methods. Examples of these methods are *in vitro* assays, omics technologies and *in silico* models (e.g., QSAR, PBPK).

Within the AFARA project, our goal is to advance standardization to stimulate regulatory acceptance of *in vitro* test assays for MDCs developed within the EU Horizon 2020 GOLIATH project (<https://beating-goliath.eu>). The models we plan to move forward include a peroxisome proliferator activated receptor gamma (PPAR γ) transactivation assay and an adipogenesis assay. By placing these assays in an Integrated Approach to Testing and Assessment (IATA), we aim to increase their predictability and relevance for assessing obesity in humans. PPAR γ is the main regulator of adipocyte differentiation. Therefore, our first goal is to determine whether activation in the PPAR γ transactivation assay can cause adipogenesis in our *in vitro* assay.

We used positive control rosiglitazone and 21 per- and polyfluoroalkyl substances (PFAS) with different physical chemical characteristics, use and occurrence in both assays. In the PPAR γ transactivation assay, PPAR γ was activated by eleven PFAS, including HPFO-DA, PFHxS, PFOA and PFOS, FBSA, PFBS, PFPrS, PFHxA, EtPFOSA and PFDA. Seven PFAS were able to induce adipogenesis. These included four PFAS that also transactivated PPAR γ (HPFO-DA, FBSA, PFBS and PFPrS), but some PFAS induced adipogenesis without being able to transactivate PPAR γ (3:3 FTCA, MeFBSA, PFPrA). And unexpectedly, five PFAS inhibited adipogenesis, of which two (PFOS and PFDA) were PPAR γ agonists.

Our study suggests that a more complex testing strategy is needed to predict the obesogenic potential of substances. This strategy should extend the number of receptor transactivation assays to screen for molecular initiating events that impact the downstream key event of adipocyte differentiation and intracellular lipid accumulation. Ultimately, this will contribute to an IATA that can be used to regulate MDCs.

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Toxicokinetic model for the transfer of aflatoxins from feed into cow's milk using a hierarchical Bayesian inference approach

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Aflatoxins are mycotoxins produced by three mold species of the genus *Aspergillus* (*Aspergillus flavus*, *A. parasiticus* and *A. minisclerotigenes*) that are frequently found in feed of farm animals, including dairy cows and thus enter the food chain. Aflatoxins display a number of different toxic effects, such as oxidative stress leading to cell death. Furthermore, the IARC has identified aflatoxins as human carcinogens. It is important to quantify aflatoxin transfer from feed into food of animal origin, such as cow's milk, as a step to understand the associated potential human health risks. Aflatoxin B1 (AFB1) is the most common aflatoxin found in feed and, among aflatoxins, displays the highest toxic potential. However, AFB1 is not transferred unmetabolized in relevant quantities into milk. In contrast to AFB1, Aflatoxin M1 (AFM1), a metabolite of AFB1, is often found in relevant quantities in milk. While AFM1 is considered less toxic than AFB1, it still exhibits significant toxic effects. Several experimental studies have demonstrated this transfer, but the resulting transfer rates, i.e., (AFM1 in milk[ng])/(AFB1 in feed[ng]), differ drastically among publications [1]. One potential explanation for this discrepancy is that the different milk yields of animals result in different excretion speeds of AFM1. Therefore, a coherent model for the transfer must take milk yield into account. One such model, a 2 compartment toxicokinetic model, was developed by van Eijkeren [2], and describes this milk yield-dependent transfer in a steady state setting. However, it is only parameterized on a very limited dataset in steady state and does not give an estimate for its potential error.

To develop a more informative model, we extended van Eijkeren's model to a dynamic setting to describe the rapid changes during the assimilation and elimination phases, and we designed and employed a hierarchical Bayesian inference approach using multiple datasets for AFM1 transfer to parameterize the model. This approach derives a distribution of model parameters for each study individually, along with an overarching distribution representing parameter variability across all studies. The overarching parameter set can then be used to predict aflatoxin transfer in milk, while providing credible intervals for predictive confidence. By including multiple studies, our approach takes into account not only animal and measurement uncertainties, but also variations due to the experimental design (e.g., different milking times), which can lead to parameter differences due to modelling imperfections. The resulting toxicokinetic model offers a quantitative way of summarizing and enhancing the findings from previous studies.

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The A-STAAAR project: Advancing neurotoxicity testing with novel strategies for drug safety and standardization

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The assessment of chemicals that have the potential of developmental neurotoxicity (DNT) relies on mammalian animal testing. As the number of chemicals being assessed for DNT increases, cost- and time-efficient toxicity testing is needed. Recently, an *in vitro* battery (IVB) OECD TG method based on the combination of individual cell-based assays has been proposed, but no attempts have been made to develop a standardized DNT testing using alternative animal models such as *Danio rerio* (Zebrafish) embryos and *Caenorhabditis elegans* (*C. elegans*). The A-STAAAR project aims to validate the DNT IVB endpoints suggested by IVB DNT method using zebrafish and *C. elegans*. This validation is conducted with reference chemicals to identify feasible endpoints in two animal models. The validated results are applied to the development of test methodologies for DNT in each animal model. Reference chemicals are selected based on their mode of action and the accuracy of the test methods is evaluated using test chemicals for which DNT has not yet been determined. This project also includes key tasks such as method reliability management and the development of standard operating procedures. The A-STAAAR project ultimately aims to establish DNT assessment markers and propose international standard test guidelines based on zebrafish and *C. elegans* as alternative models.

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Hepatotoxicity assessment of triphenyl phosphate using a liver microphysiological system

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Microphysiological systems (MPS) are promising New Approach Methodologies (NAMs)-categorized technologies. MPS is a promising technology that can mimic a physiological environment relevant to humans than conventional cell culture technology, but its usability as an alternative to animal testing for chemicals has not been well addressed. To bridge this research gap, we developed a human-derived cell line, HepG2, cultured in an MPS, to evaluate the hepatotoxicity of an organophosphorus flame retardant, triphenyl phosphate (TPhP). We optimized the culture conditions of HepG2 cells in 12 individual chips-composed MPS, which was designed and developed to facilitate toxicity assessment by increasing biological replicates, and measured the representative marker for hepatotoxicity such as lactate dehydrogenase (LDH) which were compared with the results obtained from the plate culture. And, we developed pretreatment procedures for RNA-sequencing analysis in triphenyl phosphate (TPhP)-exposed HepG2 cells and profiled the perturbed mRNAs. The optimal area and flow-rate were determined and conditions for cellular toxicity assessment were optimized such as incubation duration and embedded cell number in the MPS. In cytotoxicity assessment, we found that LDH levels were significantly decreased in the MPS after 10 μ M TPhP treatment for 48 h, which was not observed in the plate. RNA-sequencing analysis showed that hepatotoxic effect of TPhP was associated with disrupted pathways in RNA processing and spliceosome associated with liver physiology and lipid homeostasis. Furthermore, the additional analysis of MPS will be explored through quantification of chemical concentrations in the medium, with an evaluation of markers related to hepatic function such as albumin, aspartate transaminase, and urea. We showed that hepatotoxicity can be evaluated in MPS more efficiently and sensitively compare to conventional plate models and demonstrated the utility of the HepG2 cell-embedded MPS model for assessing hepatotoxicity of environmental chemicals. Additional research will follow to upgrade the MPS for long-term exposures. This will allow the MPS to better assess hepatotoxicity or liver disease due to chemical exposure and represents a true alternative to existing animal models.

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Proof-of-concept: *In vitro* skin and eye irritation testing with microbiological agrichemical formulation

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The use of bacteria in agrichemical formulations has increased to support integrated pest management. These formulations can contain chemical and microbial components, may be subject to the same testing as full chemical formulations. Skin and eye irritation testing is required for GHS classification, and these *in vitro* tests are OECD guideline compliant (TG 492 & TG 439), used as part of the testing strategy and as replacements for *in vivo* irritation tests. These *in vitro* alternative tests rely on MTT viability for evaluation, and the introduction of microorganisms may interfere with interpretation since these assays have not been validated for microbiological use.

Proof-of-concept testing was conducted to evaluate the feasibility of incorporating microorganisms into *in vitro* skin and eye irritation tests. Two formulations were tested: the first containing chemical and bacterial components; the second, a semi-blank formulation, containing only chemical components. A preliminary test determined if there was direct reaction with MTT and photometric interference. The potential interference of either formulation with the MTT endpoint was evaluated by following full assay procedures using non-viable freeze-killed tissues and assessing viability at the study's end.

In vitro eye and skin irritation tests were conducted on both formulations using Mat Tek EpiOcular™ (OCL-200-EIT) and EpiDerm™ (EPI-200-SIT) test kits. The formulations were tested concurrently, with killed control tissues incorporated into each definitive test and treated the same as viable tissues. Each assay was conducted twice. The only modification was using antibiotic-free media for initial tissue conditioning through the washing of the test material to ensure bacteria were not killed before exposure.

For comparison, an *in vivo* acute dermal irritation test was also conducted with the full formulation, following OECD 404 test guideline.

Both formulations resulted in non-irritant conclusions. No MTT or photometric interference was observed in preliminary testing. Following full testing, no MTT interference was observed on killed control tissues. The proof-of-concept results show that *in vitro* eye and skin irritation tests may be acceptable for use with bacterial formulations with little to no interference. The *in vivo* irritation test with the full formulation also resulted in non-irritant, showing concordance between the two tests for this formulation.

The bacteria used in this formulation are spore-forming. Since no MTT interference was observed at any point during testing, it is assumed the bacteria were in spore form and the incubation conditions were insufficient to reactivate them. Additional testing is required to fully validate this method using microbial formulations. Testing with known/common microorganism strains used in agrichemical formula-

tions would help gain confidence in these assays compatibility with a broader application of microbial formulations. Having formulations that show interference would help determine if any modifications to the standard assay design are required.

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Three decades of revolutionizing pyrogen testing: Human monocyte activation tests as alternatives to rabbit pyrogenicity testing

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Starting with the proposal of a PBMC-based test in 1989, groundbreaking advancements in pyrogen testing that have demonstrably reduced animal experimentation while enhancing scientific rigor in safety assessment. The development of human monocyte activation tests (MAT) has revolutionized pyrogenicity testing, which historically consumed approximately 170,000 rabbits annually in Europe alone. Four key innovations form the foundation of this work: (1) the development of the PBMC and whole blood monocyte activation tests and its cryo-variants, (2) the international validation studies establishing MAT reliability, (3) identification of lipoteichoic acid as the Gram-positive pyrogen missed by traditional Limulus assays, and (4) implementation of MAT into pharmacopoeias worldwide.

The impact on animal welfare has been substantial and quantifiable. Conservative estimates indicate annual reductions of 120,000 rabbits across Europe, with official European Pharmacopoeia statistics showing steady decreases: 170,000 rabbits (2008), 46,553 (2015), 23,885 (2020). In June 2024, the European Pharmacopoeia announced the complete deletion of the rabbit pyrogen test effective 2026, marking the culmination of this decades-long effort.

These methods have achieved broad regulatory acceptance, including integration into European, Brazilian, Indian, Russian, Chinese, and Japanese Pharmacopoeias, ISO standards for medical device testing, and FDA protocols. Commercial implementation by multiple manufacturers has established these methods as standard operating procedures throughout the pharmaceutical and medical device industries. The global Monocyte Activation Test market was valued at US\$60.7 million in 2022 and is projected to reach US\$305.3 million by 2031, demonstrating the economic viability of this alternative testing approach.

The work continues to drive progress through ongoing development of methods for material-mediated pyrogenicity, air-borne pyrogen measurements, and testing of cellular therapies, representing a comprehensive scientific strategy that successfully balances animal welfare concerns with the imperatives of human safety assessment.

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Ensuring reliable pain assessment: A meta-analytical approach to the rat grimace scale

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The Rat Grimace Scale (RGS) is widely used to assess pain in preclinical research. While its ease of use makes it an accessible tool, it is often applied in isolation without validation against other behavioral pain assays. Furthermore, it is frequently used beyond the original 48-hour window for which it was validated, possibly compromising its reliability. In the foundational study, even under ideal conditions, up to 20% of assessments were found to be potentially inaccurate. This brings to light important questions when it is used as a stand-alone assessment. When can we reliably use the RGS, and when are we at risk of missing animals in pain if we rely on the RGS as our sole measure of pain?

To better understand how the RGS is applied in research and to identify potential limitations, we have systematically reviewed studies that have utilized the RGS alongside other pain assessments. Via a meta-analytical approach, we can elucidate when the RGS aligns with other pain measures and when discrepancies could arise. This approach also incorporates a risk of bias assessment, exploring how factors such as blinding, observer variability, and experimental conditions may affect results.

Beyond assessing the performance of the RGS, this study contributes to the 3R principles. Meta-analysis can serve as a replacement method by drawing conclusions from existing data rather than requiring additional live animal testing. Our investigation ensures that refinements in pain assessment are based on high-quality, reproducible data, improving both the accuracy of pain detection and the ethical standards of preclinical research.

By integrating meta-analysis, bias assessment, and the 3R framework, we aim to clarify when the RGS can be reliably used and when caution is needed. Strengthening the evidence base for pain assessment not only enhances research quality but reinforces the ethical imperative to minimize suffering in laboratory animals.

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Mathematical modeling of the immune response to *Brucella abortus* infection: Towards reducing animal testing

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Animal trials have long been a cornerstone of immunological research, providing critical insights into immune system function, vaccine development, and therapeutic interventions. However, ethical concerns, species differences, and the need for more precise models have driven the search for alternative approaches. To address these challenges, the development of *in vivo-in silico* (iViS) models has emerged as a priority across various fields of immunology and physiology, offering significant potential benefits, including a reduction in animal trials. The use of iViS enables more complex simulations and facilitates insights that would be difficult or impossible to obtain solely through animal experiments. For example, in vaccine development, iViS allows the simulation of immune responses to distinct antigen concentrations, predicting efficacy and the need for booster doses before clinical trials. This reduces reliance on animal models while improving vaccine dose selection. In this work, we present a mathematical model for the innate immune response to *Brucella abortus* infection in C57BL/6 mice, developed within the iViS framework. The model represents populations of bacteria, antigen-presenting cells (both resting and matured), cytokines, and tissue through a system of five partial differential equations. This system enables the observation of the dynamics of these five populations across a tissue area over time. The numerical simulation was conducted for 168 hours (7 days) to represent *Brucella abortus* infection in a tissue modeled as a rectangular region of a liver from an unvaccinated C57BL/6 mouse. In this setup, 15% of the tissue represents blood vessels, and 2% represents lymphatic vessels. Preliminary numerical results demonstrate both qualitative and quantitative agreement with experimental data from the literature. The results show that areas of highest cytokine and mature antigen-presenting cell concentrations coincide with regions of pronounced infection. The model also captured a chemotactic response: resting APCs migrate toward areas with the highest cytokine concentrations, which are in turn localized to regions with greater bacterial presence. Dead tissue expands over time, closely tracking increases in bacterial concentration. Notably, regions with mature APCs show a reduction in infection spread, particularly at the infection's periphery, where APC presence at the borders actively but partially contains bacterial growth. Furthermore, by calibrating our model constants and initial conditions with experimental data, we obtained numerical results yielding an error below 6% when comparing the numerically predicted and experimentally observed dead tissue area. These qualitative and quantitative results indicate that the proposed system of equations successfully reproduces the immune system response and can potentially be used in future studies to reduce animal testing.

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Transforming safety assessment: The VHP4Safety project interdisciplinary approach

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The Virtual Human Platform for Safety Assessment (VHP4Safety) addresses the urgent need to transition from animal-based to human-based chemical and pharmaceutical safety assessment, to protect human health and the environment. This project utilizes case studies that examine adverse outcomes in various organs (e.g., kidney, brain) and incorporate human relevant scenarios, such as age, sex, disease state and lifelong chemical exposure, not covered in current animal studies.

Regulatory questions pertinent to the case studies are defined in collaboration with stakeholders, including risk assessors from industry and regulatory agencies. These questions guide the design of workflows for platform development. Adverse outcomes addressed in the include cognitive decline upon chemical induced interference of thyroid-mediated brain development, lifelong exposure to chemicals and Parkinson's Disease, and drug-induced kidney function failure.

An interoperable platform is developed offering *in silico* tools that integrate multiple human data sources, including human physiology, epidemiological and clinical data, as well as chemical characteristics and exposure scenarios. Additionally, new approach methodologies (NAMs) representing key events within adverse outcome pathways, generate new data that will be incorporated into the platform. By integrating technological innovations, including AI models such as Large Language Models (LLMs), the platform enables integration of various data sources. The workflows of the case studies form the basis of the risk assessment strategy, and preliminary results of the platform's user interfaces (minimal viable product) will be presented.

As part of the sustainability strategy, transparency, data quality, application of the FAIR principles, human relevance, reliability and training are emphasized to build trust and accelerate the transition to next-generation safety assessments, making animal testing redundant. Central to the acceptance of VHP4Safety is our commitment to an interdisciplinary approach and co-creation with multiple stakeholders, including academic, regulatory, industrial, and societal partners across the safety assessment knowledge chain. Designathons and hackathons are organized to foster a culture of open collaboration, creativity, and innovation. These events bring together experts from diverse fields to co-create the next-generation risk assessment framework and address specific challenges, benefiting from interdisciplinary expertise. Experiences and lessons learned will be shared.

The VHP4Safety project is funded by the Netherlands Research Council (NWO) under the Netherlands Research Agenda: Research on Routes by Consortia (NWA-ORC 1292.19.272).

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STE modified protocol to improve its ability to identify chemicals causing serious eye damage

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The United Nations created the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) to standardize the risk classification of chemicals. Regarding ocular damage potential, chemicals can be classified as: Category 1 (Cat 1) for severe irritants or corrosives chemicals that cause irreversible effects; Category 2 (Cat 2) for mild to moderate irritants that cause reversible effects; and No Category (No Cat) for non-irritants. This classification is based on data obtained in the *in vivo* Draize Test, performed on albino rabbits. Concerns about animal welfare, reproducibility issues, and the need for safety assessment of chemicals that may come into contact with the eyes have led to an increased demand for studies proposing evaluation strategies based on alternative *in vitro* assays. The STE (Short-Time Exposure) (OECD TG 491) is a simple test that uses a monolayer culture of rabbit corneal cells to assess the cytotoxic potential of chemicals. This assay allows classification of chemicals as Cat 1 and No Cat, without the need for further investigation. However, some chemicals, depending on the results, cannot be classified by STE as a standalone method (no prediction can be made). Besides, false negative results are a major issue in safety assessment, as they can expose the consumer to unknown risks. Thus, the aim of this study was the evaluation of chemicals with different GHS classifications by the STE assay and the proposal of a modification in its protocol that could result in an increase of its accuracy, reducing no-prediction results and the false negative rate. For this purpose, 20 substances with different GHS classifications were selected to be tested using the STE assay according to OECD TG 491 (0.05% and 5%), and at different concentrations from those recommended in guideline (0.5%, 1%, 2% and 3% for those chemicals for which prediction could not be made; and 7.5% and 10% for chemicals classified as No Cat in OECD TG 491 test. The results showed that the STE, following the OECD TG 491, was effective in classifying No Cat substances, but it was unable to classify the studied irritants (Cat 1 + Cat 2). Regarding alternative concentrations to OECD TG 491, the 3% dilution can be suggested to complementary studies as the lowest concentration allowed for the correct classification of irritants with no prediction in the first step of this study. As for the substances classified as No Cat in the first step, both test concentrations were capable of identify the irritants without generation of false positive results. As the solubility can be a problem to cell culture-based tests, the 7.5% concentration was chosen as the best one. So, we can conclude that the association of 3% and 7.5% concentrations in the STE test can result in an increased accuracy of the method.

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Flame retardants impact on human neuroprogenitor spheroids

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Flame retardants have been used as an effective tool for controlling fire spread in various scenarios. These compounds are present in items such as batteries, home furniture, and even breastfeeding devices, which leads to daily human exposure. Consequently, studying these substances has become essential to evaluate their potential toxicity, such as on human neurodevelopment. Initially, these flame retardants were used in the form of phosphorus. However, due to their toxicity, they were replaced by organophosphates, which necessitate further research to assess their potential toxicity. Tributyl Phosphate (TBP) and 2-tris-butoxyethyl phosphate (TBEP), both classified as organophosphorus flame retardants, are the primary focus of this investigation. Human-based models are crucial for evaluating adverse effects of substances on neuronal tissue. Preliminary results from our group have shown that Periodontal Ligament Stem Cells are a promising source for differentiating cells into neuroprogenitor spheroids, which are valuable for neurotoxicity testing. This model emerges as an alternative to the use of animals, aligning with the principles of the 3Rs (reduce, refine and replace). In this regard, both compounds, TBP and TBEP, were evaluated for cytotoxicity by apoptotic mechanisms. Moreover, exposing the neuronal spheroid to the compounds induced a decrease in expression of specific neural proteins, such as Nestin, SOX2 and GFAP, whereas increase of cytokines proinflammatory IL-6 and IL-8. These findings highlight the potential neurotoxic effects of TBP and TBEP, emphasizing the importance of further investigation into their impact on neuronal development. The use of Periodontal Ligament Stem Cells for neuroprogenitor spheroid differentiation provides a promising, animal-free model for evaluating the cytotoxicity of flame retardants, contributing to safer alternatives and advancing our understanding of the risks associated with these widely used compounds.

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Evaluation of bioinks for the development of a 3D bioprinted artificial skin model as an alternative to animal testing

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Introduction: Animal testing in cosmetics and pharmaceuticals faces ethical concerns, species differences, and poor translational accuracy. Despite restrictions, it remains common, but 3D bioprinting offers a human-relevant, ethical, and reliable alternative [1]. A key challenge in bioprinted skin development is selecting bioinks that ensure cell viability, proliferation, and structural integrity. This study evaluates GelMA and Nanocellulose bioinks for their potential in 3D-bioprinted human skin models as an alternative to animal testing.

Objective: To evaluate cell viability and proliferation of APSCs in GelMA and Nanocellulose bioinks over 14 days for bioprinted skin models in non-animal testing.

Materials and methods: This study was approved by the Ethics Committee of Universidad Antonio Nariño (UAN) ensuring compliance with ethical research standards.

Cell Culture & Characterization: APSCs from third molars were cultured via explant techniques in DMEM with supplements and characterized for stemness and differentiation potential.

3D Bioprinting Process: A BIO X (Cellink) bioprinter was used for extrusion-based bioprinting under standardized parameters optimized by our research group. Cells were embedded in GelMA and Nanocellulose bioinks, and constructs were incubated for 1, 7, and 14 days to evaluate their long-term viability and metabolic activity.

Viability and Proliferation Assays: Viability and proliferation were assessed using Live/Dead and WST-1 assays, with ANOVA and Tukey tests ($p < 0.05$) for statistical analysis.

Results and discussion: Cell viability and proliferation were significantly higher in GelMA bioink compared to Nanocellulose bioink at all time points ($p < 0.05$).

Live/Dead assay results showed:

- GelMA bioink: 94% viability at Day 1, increasing to 98% at Day 7, and slightly decreasing to 93% at Day 14.
- Nanocellulose bioink: 88% viability at Day 1, 90% at Day 7, and a significant drop to 82% at Day 14 ($p < 0.05$).
- WST-1 assays demonstrated similar trends:
- GelMA bioink: 92% metabolic activity at Day 1, 96% at Day 7, and 94% at Day 14.
- Nanocellulose bioink: 86% at Day 1, 87% at Day 7, and a significant reduction to 80% at Day 14 ($p < 0.05$).

These findings confirm that GelMA bioink provides better support for cell survival and proliferation, making it a more suitable material for artificial skin models. The lower performance of Nanocellulose suggests the need for further optimization, such as hybrid formulations or bioactive modifications [2].

As animal testing restrictions increase, bioprinted skin models offer a scientifically accurate and ethical alternative for toxicity and efficacy testing, improving human relevance [3,4].



Conclusions: GelMA demonstrated superior biofabrication potential, supporting high cell viability and proliferation, reinforcing 3D bioprinting as a scalable alternative to animal testing.

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Achieving a globally harmonized, animal-free regulatory framework for improved human health and environmental protection

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There is an unprecedented level of global dialogue and activity on the need to transition towards scientific approaches in regulatory testing, biomedical research and education without animals, for both scientific and ethical reasons.

With specific focus on regulatory testing, there is also a recognized urgency to develop and adopt new non-animal approaches to improve sustainability, given the need for robust and reliable safety information on

many thousands of new and emerging chemicals, which it is widely agreed the current system cannot keep pace with to provide.

The tradition of using animals in toxicity testing has cemented a conventional view of them as the “gold standard” in regulatory toxicology. However, their reliability in predicting real world responses to chemical exposure has been the subject of increasing criticism and cause for concern.

In early 2025, Lush Prize prepared a position paper on the “state of play” and current thinking on the way forward to achieve a fit-for-purpose, internationally-harmonized regulatory framework for all chemicals assessment, which is reliant exclusively on animal-free, human and environmentally relevant technologies and approaches.

The paper incorporates the feedback of a number of highly-experienced cross-regional stakeholders from industry, regulatory and NGO sectors who were consulted during a roundtable discussion hosted by Lush Prize. Additionally, a literature review includes some publications cited by stakeholders, as well as other relevant publications, guidance documents and event proceedings from a more general (but timeframe appropriate) literature review. The aim of the literature review is to provide an overview of current perspectives of the way forward to achieve regulatory acceptance of non-animal, new approach methodologies (NAMs) highlighting publications on relevant topics, including international roadmap initiatives, legislative progress and related activities on how new methods can be validated, quantified and approved as part of a redefined “gold standard” which is entirely animal free.

The overriding key message from our study is that collaboration and communication between all from the outset is essential for change. Communication must be multi-directional to achieve regulatory acceptance of non-animal approaches and continue to build confidence and trust in both phase-in of new methods and phase-out of animal tests. All requirements for the way forward are critically dependent on an effective communications strategy which should include robust change management and implementation measures of success, in order to see full impact and a transition towards animal-free regulatory testing.

Setting deadlines to end animal testing have been met with resistance and reluctance but are still considered by many to be the only way forward to accelerate the change needed, especially when considered the successful drive in non-animal methods in response to the EU cosmetics animal testing bans.

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Development and characterization of a hepatic spheroid model integrating Kupffer-like macrophages for *in vitro* toxicity testing

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Systemic toxicity is a critical concern in drug and chemical safety assessment. It arises from both nonspecific cytotoxicity and tissue-specific effects post absorption. Currently, its evaluation relies on animal testing, particularly the acute oral toxicity test used to determine the median lethal dose (LD50) in rodents. However, this approach presents significant ethical concerns, high variability, and limited human relevance. As regulatory agencies shift towards New Approach Methodologies (NAMs), there is a growing need for advanced *in vitro* models that can improve toxicological predictions while replacing or reducing animal use.

The liver plays a central role in systemic toxicity, as it is responsible for metabolism and detoxification. Many xenobiotics undergo bioactivation in hepatocytes, leading to the formation of reactive metabolites

that contribute to hepatotoxicity and systemic effects. Drug-induced liver injury (DILI) remains a major cause of drug withdrawal and a key factor in preclinical safety evaluations. Human-relevant *in vitro* liver models, such as 2D hepatocyte cultures, 3D spheroids, and liver-on-a-chip systems, have demonstrated improved physiological relevance compared to animal models, providing valuable insights into metabolism, toxicity, and inflammatory responses. However, further advancements are required to enhance their predictive accuracy and reproducibility in regulatory toxicology.

This study presents the development of a 3D hepatic spheroid model integrating HepG2 hepatocytes and THP-1-derived macrophages resembling Kupffer cells, crucial mediators of liver inflammation and drug-induced toxicity. This model aims to replicate essential liver functions and immune interactions relevant to systemic toxicity. The initial phase of this project focuses on: (1) differentiating THP-1 cells into macrophage-like Kupffer cells, (2) achieving stable spheroid formation, and (3) conducting functional and morphological characterization, including viability, protein synthesis, hepatic function, amino acid metabolism and metabolic enzyme activity. Reference compounds with well-documented human toxicity profiles were tested to assess the model's predictive capacity for hepatotoxicity evaluation.

By providing a more physiologically relevant platform, this model supports the transition to *in vitro* alternatives aligned with regulatory requirements. Future integration with computational tools and multi-organ systems could further enhance its application in chemical risk assessment, reinforcing the potential of 3D hepatic models to replace traditional animal-based methods in toxicity testing.

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A comparative study of γ radiation effects on chitosan / bioactive glass / spions scaffolds

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Injuries to the peripheral nervous system result in neuropathies that impair essential functions regulated by the nervous system. Current peripheral nerve repair techniques allow only partial structure recovery, highlighting the need to explore more effective alternatives. In tissue engineering, scaffolds are widely used for tissue regeneration, and the combination of chitosan, bioactive glass and superparamagnetic iron oxide nanoparticles (SPIONs) provides material stability, biodegradability and guidance for cellular growth. Sterilization processes are crucial to maintaining these properties without the risk of contamination or functional impairment. This study aimed to chemically characterize the three-phase composite material before and after sterilization by ionizing γ radiation, assessing possible chemical structure changes induced by irradiation doses of 15 kGy and 25 kGy in a gamma chamber, according to ISO 11.137- Part 8. Structure modifications were analyzed using Fourier transform infrared spectroscopy (FTIR) with attenuated total reflectance (ATR) in the wavelength range of 700 and 5000 cm⁻¹, performing 64 scans per sample. In addition, as an alternative approach to animal testing, the cytotoxic effects of the composite were evaluated with NIH-3T3 cells investigated using MTS as a staining reagent for viable cells. The FTIR spectra indicated the preservation of the characteristic chemical bond bands in the generated spectra, without significant changes in their position. However, after irradiation, variations in peak intensities were observed, suggesting interactions between the material's polymeric chains. The indirect cytotoxicity assays demonstrated high cell viability in both irradiated and non-irradiated samples, confirming the composite's biocompatibility. These results demonstrate a promising potential for nerve regeneration applications.

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Two decades of animal testing in the EU: Tracking progress ahead of the 2026 phase-out roadmap

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The European Commission's roadmap to phase out animal testing for chemicals, set for implementation from 2026, marks a pivotal step towards modernizing science and eliminating animal suffering. In response to growing calls for action, the Commission committed to developing a comprehensive plan to eliminate animal testing for chemical safety assessments in the European Union (EU). This roadmap will outline specific actions, milestones, and timelines to replace animal testing, with an emphasis on accelerating the adoption of non-animal methods.

Ahead of implementation of the chemicals roadmap, this poster reviews the progress made in reducing animal testing in the EU over the past 20 years, focusing on key factors such as the primary purposes of testing, the key regulations driving regulatory testing, the countries with the highest animal use, and trends over time. Since 2002, a very modest reduction in animal testing across the EU is evident, with a particularly notable decline between 2005 and 2015. However, the rate of reduction has slowed in recent years. If these trends continue, animal testing could persist well into the 22nd century.

Non-animal methods must be adopted more rapidly; the Commission's roadmap presents an opportunity for a meaningful decline to finally be realized. With the stated aim of ending all required regulatory tests on animals for industrial chemicals, pesticides, and human and veterinary medicines, the roadmap aims to accelerate the integration of non-animal alternatives into the EU's regulatory frameworks.

Looking ahead, tracking these trends in animal use and uptake of non-animal methods will be a key metric for evaluating the success of the Commission's roadmap.

We will evaluate the actions outlined in the draft chemicals roadmap, available from the summer of 2025, in light of the observed trends in animal use and will make recommendations for the most effective implementation of the roadmap from 2026 and beyond. This work is vital for advancing both ethical and scientifically innovative practices, ensuring that animal testing finally becomes a thing of the past.

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10 animal tests with non-animal alternatives – Why haven't they stopped?

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It is often assumed that animal tests with non-animal replacements are no longer conducted, or at least are rarely. However, in reality, these tests persist and may even increase long after suitable alternatives have been established. To address this issue, Cruelty Free International has tracked several animal tests over the past eight years to assess the scale and causes behind this phenomenon.

The Replace Animal Tests (RAT) list highlights ten regulatory animal tests that continue to be used, despite having scientifically valid non-animal alternatives available. These tests include rabbit pyrogen, skin and eye irritation, skin sensitization, antibody production, and various batch safety tests.

In the latest version of the RAT list, we present updated statistical evidence on the ongoing use of these tests and the status of their non-animal replacements across regions, including Europe, North America, South America, and Asia. We also highlight the main barriers preventing the widespread uptake of non-animal approaches. Key factors contributing to the persistence of these tests include the desire for international harmonization before full replacement, unclear regulatory requirements, and insufficient monitoring and enforcement.

The RAT list is a useful tool to highlight animal tests that are long overdue for replacement, track trends across sectors and regions, and expose the systemic issues hindering the adoption of non-animal methods. By documenting these tests, the list serves as an essential resource for educating stakeholders and advocating for the completion of the replacement process on both scientific and moral grounds.

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The prevalence of reported gavage incidents in repeated dose toxicity studies involving rats conducted between 2004-2023

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In animal-based toxicology studies, oral gavage is the predominant technique for administering compounds directly into the stomach. Recognized as a stressful procedure for animals, gavage is associated with accidents and “gavage-related reflux” (GRR), which may lead to respiratory distress and/or death. This study estimates the prevalence of incidents (deaths and/or non-fatal clinical signs) attributed to the gavage procedure in a representative sample of repeated dose toxicity test reports from the last 20 years, sourced from the ECHA CHEM database of REACH-registered chemicals.

In 21% of studies, at least one death or clinical sign in rats was attributed to the gavage procedure in the study report. An additional 16% of studies reported clinical signs associated with GRR in the animals, with no other adverse effects, but without attribution to the gavage procedure. Among those studies with incidents attributed to the gavage procedure, 90-day studies were three times more common than 28-day studies; incidents were also more likely to have occurred with the test substance rather than the control, with highly viscous substances and in more recently conducted studies. There was no association between the number of studies with a gavage-attributed incident and the strain, size, or age of the rats, the dose volume or the testing of irritant substances.

These findings suggest that incidents attributed to gavage are more prevalent than previously thought and that a review of the gavage procedure is urgently needed.

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Conformation of Argentinian network of alternative methods (RAMA: Red Argentina de métodos alternativos): An experience

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The RAMA aims to promote close collaboration between technicians, regulators and researchers, in order to develop and implement methods that refine, reduce or replace the use of animals in experimentation, without compromising the quality and accuracy of the results obtained in the tests carried out.

At the beginning of 2013 in Argentina the development and implementation of alternative methods was in an incipient state, unlike other countries in the region such as Brasil. But the creation of the Ibero-Latinamerican network of alternative methods at Latinfarma 2013 (CUBA) meeting, try to promote this area of expertise in Argentina.

In this sense, next year Argentinian and international scientific societies related to animal experimentation (AACyTAL and FESSACAL) organized the 1st workshop on alternative methods in the country in order to promote and develop this area in the society and facilitate the participation of scientists in the Regional Exchange Programs of training “*Theoretical-Practical Course for utilização de métodos alternativos ao uso de animais in vitro para avaliação do potencial of ocular irritation and corrosão of chemical substances*” organized by CABBIO through INMETRO/Rio de Janeiro, Brasil. Although the limited resources, RAMA offer different activities such as conferences, workshop, seminars and lectures during the next years. Particularly, it was involved in the organization of the first congress related to 3 Rs in Argentina in 2018 (COLAMA III). In fact, RAMA members have participated in the different editions of COLAMA since 2012 (Brazil, Cuba, the aforementioned Argentina, Mexico and Colombia), collaborating through the organization and scientific programs. Interestingly, despite the pandemic, the network remained active by organizing virtual seminars with national researchers working in this issue and also international leaders that could share their particular experiences in the formation of networks in their countries. Lastly, a remarkable achievement of this network has been the recently incorporation of a chapter in the Argentinian Pharmacopoeia (Seventh edition, second Supplement) 1092 (*Métodos alternativos al uso de animales con fines regulatorios*, IF-2023-140107866-APN-DFYGR#ANMAT), introducing the main concept of alternative methodologies to the use of animals for regulatory purposes, promoting and encourage initiatives to implement the principles of 3Rs in both public and private spheres

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ICAPPP: Advocating for animal protection in pharmaceutical testing

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The International Council on Animal Protection in Pharmaceutical Programs (ICAPPP) is a global organization dedicated to advancing animal protection within international pharmaceutical testing guidelines developed by the International Council for Harmonisation (ICH) and the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH).

Founded in 2005, ICAPPP was created as an offshoot of the International Council on Animal Protection in OECD Programmes (ICAPO). Since gaining “invited expert” status in 2002, ICAPO has contributed to over 120 guidelines developed by the Organisation for Economic Co-operation and Development (OECD), guidelines which enable the widespread use and regulatory application of non-animal testing methods and risk assessment approaches. ICAPO also provides financial and expert support for key initiatives at the OECD and helps shape regulatory strategies which maximize reliance on non-animal testing.

Although the ICH and VICH currently do not formally recognize external stakeholders, ICAPPP has contributed to harmonization efforts by providing feedback on draft guidelines and attending regional update meetings. Our advocacy has led to meaningful changes, such as stronger language prioritizing non-animal methods and the 3Rs principles, clearer guidance on the use of non-animal methods and the removal of language promoting outdated or inhumane testing practices. Additionally, ICAPPP holds Interested Party status with the Committee for Veterinary Medicinal Products (CVMP) at the European Medicines Agency (EMA), reflecting our active engagement in the regulatory process.

We believe an international voice for animal protection in the ICH and VICH frameworks is crucial, and while we continue to urge both bodies to formally include us, we remain committed to pushing for meaningful changes within our current capacity. We encourage anyone interested in advancing animal welfare in pharmaceutical testing to contact us. Additionally, we welcome opportunities to collaborate with ICH and VICH Members and Observers who are looking to expand their stakeholder engagement. ICAPPP is ready to offer our expertise and support as we work together toward a more ethical and scientifically advanced future.

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Reviewing the use of two species in toxicity testing for human pharmaceuticals

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Regulatory agencies worldwide typically require toxicity testing in two animal species before a drug can progress to clinical trials – usually one rodent and one non-rodent species, such as dogs or primates. This two species approach is based on the assumption that data from the second species provides an added layer of safety by identifying adverse effects not detected in rodent tests. However, growing evidence challenges this assumption, suggesting that a single-species approach could reduce animal suffering without compromising human safety.

We are planning a hybrid workshop series at Johns Hopkins University, where participants can attend either in-person or virtually, to reassess the need for two species testing, especially in light of advancements in new approach methodologies (NAMs).

Part One will focus on the feasibility of transitioning from the traditional two-species approach to a single-species approach in toxicity testing. Participants will examine the current International Council for Harmonisation (ICH) M3 (R2) guideline, which recommends two-species testing for small molecule pharmaceuticals, and explore conditions under which non-rodent studies, in particular, could be waived – especially for sub-chronic and chronic studies. They will also assess whether such an approach could still ensure adequate protection for human health. Part Two will explore the potential for NAMs to replace the need for a second species in toxicity assessment, highlighting recent advancements in science and technology, including microphysiological systems, that could enable more humane and efficient testing practices.

We are calling on experts in drug safety, toxicology, regulatory science, NAMs development, and academic research with specialized expertise in these areas to join us in this important dialogue. Participants will have the opportunity to engage, review the latest evidence, and help shape recommendations for regulatory bodies such as the International Council for Harmonisation (ICH). The goal is to explore how regulatory frameworks can be updated to reflect current scientific understanding as the science continues to evolve.

We invite you to contribute your expertise and help shape the future of drug safety testing. For more information, please contact us directly.

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The importance of stakeholder collaboration for legislative change: The ban on animal testing for cosmetics in Chile

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The path to ending animal testing for cosmetics in Chile began in 2016 when two Chilean organizations, “ONG Te Protejo” and “No Más Vivisección,” alongside a bipartisan group advocating for animal rights, introduced a bill to ban animal testing for cosmetics. This initial effort emerged within a context where awareness of cosmetic animal testing was gaining momentum in Chilean society, particularly through the growing presence of cruelty-free certification in the local market. Consumer interest in ethical consumption played a key role in shaping the discussion around the bill.

The proposed legislation made modifications to the Chilean Health Code, which required its first legislative discussion to take place in the Congress’ Health Commission. This presented a significant challenge, as the commission had to balance various pressing public health priorities. Despite these challenges, advocacy efforts continued, and in 2017, ONG Te Protejo, in collaboration with Humane Society International, launched the “Be Cruelty Free Chile” campaign. This initiative was key in consolidating public support for the bill and demonstrated the importance of articulating the different stakeholders to ensure that legislative efforts aligned with local needs.

Years of public awareness campaigns, a methodic lobbying strategy, and continuous engagement with stakeholders contributed to widespread support for the proposed legislation. However, ensuring the bill remained on the legislative agenda required constant monitoring and persistence. By 2020, nearly five years after the introduction of the bill, a strategic decision was made to submit a new bill. Throughout the years of campaigning and legislative work, several countries enacted similar bans, allowing them to learn from their challenges and refine their approach and to harmonize with international regulations. This resulted in the introduction of a new bill in December 2020, which secured support from both national and international cosmetic companies.

The updated bill advanced through legislative discussions, supported by continued lobbying efforts and collaboration with industry stakeholders, in addition to strong support from citizens. In the final stages of legislative review, the bill received public approval from the Chilean Cosmetics Chamber and the Public Health Institute. This broad consensus enabled its unanimous approval in its final vote, marking a significant milestone in Chile’s legislative progress towards ethical cosmetic practices and animal rights. This case highlights the crucial role of coordinated efforts among diverse stakeholders in enabling legislative reform.

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Mixture toxicity assessment of 1,2-benzisothiazol-3(2H)-one (BIT) and co-existing chemicals in consumer products using *in vitro* human cells

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Humans are routinely co-exposed to complex chemical mixtures through consumer products. However, traditional toxicity assessments typically focus only on single chemicals, limiting our understanding of mixture toxicities. This study aimed to identify chemical mixtures in consumer products and evaluate their potential systemic toxicity using an *in vitro* approach. As a case study, we focused on 1,2-Benzisothiazol-3(2H)-one (BIT), a preservative widely used in consumer products. We gathered data on chemical components in products from Ecolife, a Korean consumer product database. To enable a component-based analysis, we identified binary, ternary, and quaternary mixtures present in these products. Mixture combinations for toxicity screening were selected based on their prevalence in consumer products and chemical characteristics: preservatives and potential allergenic compounds. Since the exact mixture composition ratios in consumer products are unknown, various combinations such as equal-proportion and equitoxic mixtures were tested to cover multiple possible scenarios. Cytotoxicity was assessed using four human cell lines: Beas-2b, E6-1, HepG2, and SH-SY5Y. Predicted cytotoxicity of mixtures was calculated using the concentration addition (CA) and independent action (IA) models, based on parameters derived from dose-response curves of individual chemicals. The effect of the mixtures was evaluated by comparing predicted values with experimental results through the model deviation ratio (MDR). Ultimately, specific combinations containing BIT that exhibited synergistic effects in consumer products were identified. This case study proposes a strategy for assessing complex chemical mixture in consumer products. The findings highlight the importance of considering mixture toxicity in consumer product risk assessments, as this aspect may be overlooked when assessments focus solely on single chemical toxicities. Moreover, the *in vitro* method proposed in this study allowed for efficient testing of various combinations, facilitating more comprehensive and effective toxicological evaluations.

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In silico molecular docking-based screening of plastic additives leaching from plastic polymers

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Plastic additive chemicals represent emerging contaminants due to their potential release from polymer matrices, posing significant toxicological risks. To efficiently prioritize these chemicals for regulatory action, we utilized molecular docking simulations, an innovative new approach methodology (NAM), to systematically predict binding affinities between plastic additive chemicals and critical human nuclear receptors. Based on a previously conducted systematic evidence mapping (SEM) highlighting substantial gaps in empirical knowledge regarding the leaching potential and toxicity of numerous plastic additives, we selected 150 functional additives registered under REACH from the European Chemicals Agency (ECHA) database. These additives were screened against seven nuclear receptors (ERs, AR, GR, PPARs, FXR, PXR, CAR), which are known molecular initiating events (MIEs) associated with adverse outcome pathways (AOPs). Our analysis identified the constitutive androstane receptor (CAR) as the most responsive receptor, with 67 additive chemicals demonstrating significant binding potential to at least one receptor. Notably, top-ranked additives were frequently utilized in widely used polymers such as polyvinyl chloride (PVC) and polyethylene terephthalate (PET), underscoring their potential environmental and health relevance. Even though these additives have potential to contribute significantly to the hazards of microplastics, most are frequently used without sufficient regulatory restrictions. The integration of computational toxicology and evidence mapping approaches presented herein effectively supports hazard identification and prioritization, guiding future empirical assessments and regulatory strategies for plastic-associated chemicals. Therefore, we suggest that further assessment of these additive chemicals and their corresponding plastic polymers under co-exposure conditions should be conducted to comprehensively address their combined risks.

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Accelerating global adoption of new approach methodologies (NAMs): The European Research Area's 2025 initiative for biomedical research and regulatory testing of medicinal products and medical devices

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The European Research Area (ERA) is poised to launch a comprehensive action in 2025 aimed at accelerating the development, validation, and uptake of New Approach Methodologies (NAMs) across all stages of biomedical research and the regulatory testing of medicinal products and medical devices. This initiative seeks to harmonize efforts among EU Member States and invites global collaboration to enhance the efficacy and ethical standards of biomedical research.

In 2022, approximately 8.5 million animals were utilized for scientific purposes within the European Union, with 72% engaged in basic, translational, and applied research, and 13% in regulatory testing – 78% of which pertained to medicinal products and medical devices. Addressing this substantial animal usage, the ERA's forthcoming action targets the development and adoption of NAMs, aiming to reduce reliance on animal models by providing scientifically validated alternatives that meet regulatory requirements. It will establish an EU-wide forum encompassing ministries, regulatory agencies, research funding organizations, academia, industry representatives, Contract Research Organizations (CROs), SMEs, and startups. This collaborative platform will align national and regional policies to expedite NAM devel-

opment, validation, acceptance, and implementation. Four specialized Working Groups (WGs) will be deployed to address critical facets of NAM integration:

WG1: Development of NAMs and common European infrastructures. It will identify opportunities for NAM development in specific disease areas and safety assessments. It will promote the establishment and sustainability of European infrastructures providing NAM resources, such as biobanks and computational models.

WG2: Validation/qualification, acceptance, and uptake of NAMs. It will define criteria facilitating NAM uptake in research and regulatory contexts. It will prioritize and implement the validation and qualification of mature NAMs for regulatory testing.

WG3: Education and training. It will map and assess existing education and training programmes on NAMs and the 3Rs principles. It will develop common modules in partnership with educational institutions to engage current and future professionals, ensuring widespread competency in NAM application.

WG4: Openness and awareness. It will develop policies to improve research transparency, including open access to NAM protocols and publication of all animal studies. It will share best practices among ethical committees, funding bodies, reviewers, and regulators to maintain up-to-date awareness of NAM advancements. It will engage civil society and patients to raise awareness about the benefits and challenges of various research methodologies in developing safe and effective treatments.

The ERA recognizes that the challenges and opportunities presented by NAMs are not confined to Europe. Global collaboration is essential to harmonize standards, share best practices, collectively advance the field of biomedical research, and ensure acceptance by regulators worldwide. We invite international stakeholders to join this initiative, contributing to a global effort to enhance research methodologies, reduce animal usage, and accelerate the development of safe and effective medical innovations.

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Inter-laboratory validation of the ToxTracker assay for mechanistic genotoxicity assessment

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ToxTracker is a flow-cytometry based mammalian cell reporter assay that accurately predicts the genotoxic properties of compounds with high accuracy. By evaluating the induction of various reporter genes that play a key role in cellular pathways relevant for genetic toxicology, ToxTracker provides insight into the chemical mode-of-action (MoA), thereby discriminating direct-acting genotoxicants from cytotoxic chemicals that induce DNA damage secondarily. To investigate how ToxTracker may complement the standard battery of *in vitro* genotoxicity assays, a comprehensive interlaboratory validation trial was conducted according to OECD Guidance Document 34.

The primary objectives of this prospective validation study were establishing transferability and reproducibility of the assay and confirming the ability of ToxTracker to correctly classify genotoxic and non-genotoxic compounds. Additionally, reproducibility of the assay to predict genotoxic MoA was confirmed across participating laboratories and the applicability of ToxTracker for regulatory applications and how it can be applied to improve the *in vitro* genotoxicity testing strategies was explored.

In the validation trial, seven laboratories tested 64 genotoxic and non-genotoxic chemicals that together cover a broad spectrum of chemical spaces. The within-laboratory reproducibility (WLR) was up to 98% (73%-98% across participants) and the overall between-laboratory reproducibility (BLR) was 83%. This trial confirmed the accuracy of ToxTracker to predict *in vivo* genotoxicants with a sensitivity of 84.4% and a specificity of 91.2%

From this validation trial we concluded that ToxTracker is a robust *in vitro* assay for the accurate prediction of *in vivo* genotoxicity. With information on the MoA of chemicals that is provided by the assay and considering its robust standalone accuracy, ToxTracker is a valuable addition to the standard battery of genotoxicity assays that is applied for regulatory applications and has the potential to mature into one of the mammalian cell assay options as a genotoxicity test battery component.

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Integrated approach to testing and assessment (IATA) for developmental neurotoxicity to prioritize per- and polyfluoroalkyl substances

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Per- and polyfluoroalkyl substances (PFAS) are widely detected in consumer products and the environment, raising concerns due to their persistence and potential developmental neurotoxicity (DNT). Epidemiological studies have linked PFAS exposure to cognitive and behavioral deficits, necessitating a systematic hazard characterization approach. This study employs an Adverse Outcome Pathway (AOP)-based Integrated Approaches to Testing and Assessment (IATA) framework to assess PFAS-induced DNT.

Our methodology consists of three key steps: First, we developed a PFAS DNT AOP using AOP-Wiki and the Comparative Toxicogenomics Database (CTD), identifying key molecular initiating events (MIEs) and downstream key events (KEs). Second, we collected data from *in vivo*, *in vitro*, and high-throughput screening (HTS) studies to populate AOP components. For key events lacking sufficient data, we generated *in vitro* experimental data using neural stem cells to supplement the dataset. Finally, we employed a Bayesian Network (BN) approach to quantitatively assess and compare key events (KEs) within the AOP, allowing us to prioritize commonly used PFAS based on their developmental neurotoxicity risk.

Findings indicate that PFAS acts as a thyroid receptor antagonist, disrupting hormone signaling critical for brain development. This perturbation led to KE activation, including neuroinflammation, oxidative stress, and apoptosis, culminating in neurodevelopmental deficits. Notably, PFAS exposure reduced brain-derived neurotrophic factor (BDNF) levels, impacting neuronal survival. The AOP-based analysis suggests potential implications for learning disabilities, aligning with observed epidemiological trends. Notably, PFOS exhibited stronger developmental neurotoxicity compared to short-chain PFAS, such as PFBS.

This study demonstrates the utility of an AOP-informed IATA framework in hazard characterization, providing a mechanistically anchored approach for evaluating PFAS neurotoxicity. These findings reinforce the importance of integrating mechanistic data into chemical risk assessment and highlight the broader applicability of AOP-based IATA strategies in regulatory toxicology.

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Transformation of maple toxins hypoglycin A, hypoglycin B and methylene cyclopropyl glycine in a ruminal batch culture model

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In animal nutrition research, batch culture refers to a sample of the microbial community in its natural medium, taken from a target digestive compartment of a donor animal, inoculated and supplied with energy and macronutrients through substrates (fed batch culture). *In vitro* models that simulate ruminal fermentation are increasingly important to reduce or replace animal testing. For risk assessment purposes, models can be used to investigate the transformation or the metabolism of a potentially undesirable substance such as a plant toxin, which is part of assessing any potential transfer into animal-derived products. In this study, the transformation of the maple toxins hypoglycin A (HGA), hypoglycin B (HGB) and methylene cyclopropyl glycine (MCPrG) was investigated in ruminal fluid batch cultures and the effect of these toxins on the batch culture microorganisms was measured using microbial metabolites. The experiment comprised four 48 h-runs in the ANKOM RF Gas Production System using ovine ruminal fluid. The fermenters either contained 200 mg of a substrate mixture (80% cellulose, 20% starch; CSM), CSM and 1.5 mL of a solution of pure toxins (500 ng/mL HGA and 500 ng/mL MCPrG; PCM) or CSM and 100 mg sycamore maple seeds (SMS) and each fermenter was inoculated with 30 mL of ruminal fluid and buffer at 1:2 (v/v). For control, autoclaved ruminal fluid was incubated with CSM, PCM and SMS, respectively. Samples were taken from the liquid phase of the fermenters after 0, 1, 2, 4, 8, 24 and 48 h and analyzed for the toxins and their metabolites using liquid chromatography-tandem mass spectrometry (LC/MS-MS). Microbial activity was assessed using gas production, short chain fatty acids and ammonia concentration. The pH and redox potentials were measured. With PCM, HGA and MCPrG rapidly decreased until 24 h of incubation ($p < 0.05$). With SMS, the initial concentrations were 4.7 ± 1.4 µg/mL HGA, 19.9 ± 5.41 µg/mL HGB and 1.2 ± 0.33 µg/mL MCPrG. A rapid conversion of HGB to HGA was revealed, accompanied by a progressive decrease in HGA concentration ($p < 0.05$). The concentration of MCPrG was constant until 4 h and decreased afterwards ($p < 0.05$). With SMS, HGA and MCPrG concentrations of 5.6 ± 1.5 and 0.32 ± 0.090 µg/mL remained stable until 48 h, respectively. The HGB to HGA conversion and transformation of HGA and MCPrG also occurred in autoclaved ruminal fluid. Gas production and microbial metabolite concentrations were higher in SMS compared to CSM and PCM ($p < 0.05$), which shows the seeds were used as an additional substrate. Batch culture tests with potentially harmful substances may provide information on whether the target substance can basically be metabolized in the rumen environment or is not. According to the 3R principles, *in vitro* models applied as preliminary screening tools allow more efficient animal experiments and a reduction of animal numbers.

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Application of animal product-free alternatives in the performance of the *in vitro* micronucleus (IVMN) assay

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Whilst human *in vitro* assays for genotoxicity testing are well established, they still rely on animal derived supplements, such as fetal bovine serum (FBS) or rat liver homogenate fractions (S9). Ethical concerns and batch-to-batch variation of these supplements are driving the move towards defined, more human relevant solutions. Chemically defined serum replacement (CDSR) is commercially available, however transferring to these more human relevant alternatives is challenging as cellular characteristics are often altered, particularly related to proliferation meaning a direct substitution with serum with the same assay methodology without taking into account for changed cell behavior is not possible. Previously, we have demonstrated that TK6 cells can be successfully transitioned from FBS to animal-product free medium without any significant effect on their cell health, morphology or phenotype. The aim of this study was to evaluate the functionality of new animal product free TK6 in a genotoxicity test: *in vitro* micronuclei (IVMN) assay and compare their performance with standard FBS cultured TK6 cells.

Human lymphoblastoid TK6 cells cultured in either FBS- or CDSR-supplemented medium were utilized in the IVMN assay according to OECD guideline TG487. Mitomycin C (MMC), methyl methane sulphonate (MMS), vinblastine and colchicine were used as test chemicals to induce DNA damage. Following short (3 h) or long (24 h) treatment, the formation of micronuclei (MN) was monitored. Replicative index (RI) was used to assess cytotoxicity in the presence of cytochalasin-B (Cyto-B). The induced MN formation in cells cultured in FBS containing media was compared to MN induction in cells cultured in CDSR supplemented medium.

TK6 cells cultured in serum free media under-go a lag phase in their growth profile, and adaptation of the methodology to include an extended recovery period (1.5-2 cell cycles) was essential for MN formation following treatments. Following these modifications, it was reported that the MN induction in TK6 cells for the untreated controls were within the historical control ranges (0.4-1.5%) for both (FBS vs CDSR) culture conditions. Furthermore, a clear dose dependent increase in MN induction was recorded for all tested chemicals in serum free media and was comparable to MN frequency observed in cells cultured in FBS containing media.

In conclusion, the equivalent performance of the animal product-free *in vitro* assay against the standard TG487 test has been demonstrated. This study supports the use of serum free media for routine genotoxicity testing, as well as building confidence in animal product free alternatives demonstrating their fit-for-purpose. Moreover, outputs of this project provide researchers with animal product-free tools and approaches applicable to over 95% of *in vitro* cell culture assay methodologies available.

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In vitro production of cattle and pig embryos as models for evaluating cadmium-induced developmental toxicity

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While many areas of research are showing a decrease in the use of experimental animals, this trend does not extend to developmental toxicity studies. New methods are needed to predict and assess harmful effects using ethically sound approaches that allow extrapolation between species. The aim of this study was to evaluate a multi-species *in vitro* embryo production (IVEP) model using gametes from cattle and pigs, as a step to minimize animal use. Cadmium (Cd), which is toxic and bioaccumulates in the ovary, was added to the oocyte maturation medium to mimic ovarian exposure. The concentration was based on previous studies.

Ovaries were retrieved from an abattoir, and oocytes (N = 1618 in cattle and N = 2907 in pigs) were matured *in vitro* in the presence of Cd (2 µg/mL) dissolved in 1 µg/mL DMSO, DMSO alone (1 µg/mL), or a control medium without addition. IVEP followed standardized protocols. For cattle, the maturation time (which was also the treatment time) was 22 h, while for pigs it was 44 h. After maturation, oocytes were fertilized with frozen semen from a single bull or boar, and presumed zygotes were cultured until day 6 (pigs) or day 8 (cattle). Evaluation of the oocytes and different embryo stages was performed 2 days after fertilization for the first cleavage divisions, and for blastocyst development on day 5 and 6 (pigs) or day 7 and 8 (cattle). Statistical analysis was performed using mixed-effects logistic regression with binary distribution and replicate as a random factor (Glimmix procedure of SAS 9.4, Milltown, USA). Data are presented as percentages calculated from the total number of treated oocytes ± standard error of the mean, in the order: Cd, controls, and DMSO.

There were no effects of Cd on cattle embryos at first and second cleavage (1st cleavage: 72 ± 0.04, 73 ± 0.04, 72 ± 0.04; 2nd cleavage: 65 ± 0.03, 67 ± 0.03, 64 ± 0.03). However, in pig embryos, Cd reduced the proportion of surviving embryos compared to controls (p < 0.01) at both first and second cleavage (1st cleavage: 36 ± 0.02, 46 ± 0.03, 37 ± 0.02; 2nd cleavage: 27 ± 0.02, 33 ± 0.02, 29 ± 0.02). Cd-treated cattle blastocyst count was significantly reduced compared to controls on both day 7 (p < 0.05, 25 ± 0.02, 32 ± 0.03, 26 ± 0.03) and day 8 (p < 0.01, 30 ± 0.03, 40 ± 0.04, 36 ± 0.03). For pig embryos, there were no significant differences on day 5 (8 ± 0.01, 9 ± 0.01, 9 ± 0.01) or day 6 (13 ± 0.01, 16 ± 0.01, 12 ± 0.01).

There was no effect of DMSO on cattle embryos. However, in pig embryos, DMSO reduced the proportion reaching first cleavage compared to controls (p < 0.01) but had no effect on second cleavage or later development. This suggests that DMSO can alter Cd effects in early pig embryos, but cattle embryos appear less sensitive.

In conclusion, IVEP is suitable for studying harmful chemical effects on oocyte maturation and embryo development without animal use. However, species differences and vehicle effects must be considered when interpreting results.

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Assessing the potential of per- and polyfluoroalkyl substances in developing and adult human renal cells

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Per- and polyfluoroalkyl substances (PFAS) are identified as prominent environmental contaminants. These compounds are primarily cleared by the kidney where they may affect renal function and renal repair mechanisms. This study will evaluate whether PFAS adversely affect the developing and adult human renal cells, employing human induced pluripotent stem cell (iPSC)-derived proximal tubular-like cells (PTLs). Parameters such as attachment, proliferation, differentiation and differentiated functions are assessed. Additionally, iPSC-derived endothelial and podocyte-like cells will be assessed as other potential target cells. Our preliminary results suggested that cell viability and morphology are affected with specific PFAS compounds at micromolar concentrations, while others had no observed effects. These results further emphasize the need to characterize these compounds where human and environmental exposure is suspected.

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New approach methodologies (NAMs) for understanding the impact of endocrine disruptors in the framework of the HYPIEND project

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Endocrine-disrupting chemicals (EDCs) are substances that mimic, block or interfere with hormones in the body's endocrine system. They have been linked to numerous adverse human health outcomes, including the abnormal function of the hypothalamus-pituitary (HP) axis, a complex system of neuroendocrine pathways and feedback loops essential to maintaining physiological homeostasis. Alteration of the HP axis is especially relevant in the early stages of life because it can cause developmental alterations, psychosocial difficulties, and behavioral disorders.

The objective of the Horizon Europe HYPIEND project is to understand the effects of EDCs co-exposure on the function of the HP axis by combining different New Approach Methodologies (NAMs) together with animal and interventional studies aimed to minimize the exposure and the consequences of EDCs on the neuroendocrine system during the perinatal and pre-pubertal stages.

NAMs have been proposed as an affordable and faster alternative to animal experimentation for testing chemicals safety and are a priority for toxicological research and for regulatory bodies world-wide. The NAMs to be used in the HYPIEND project include cell-based *in vitro* models, *in silico* models (combining QSAR methodologies and structural analysis), *Danio rerio* models, and new models of organoids-based organ-on-chip recapitulating the HP axis. By integrating these methodologies, HYPIEND will provide a comprehensive and mechanistic understanding of EDCs-induced disruptions in neuroendocrine function.

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Zebrafish myelin sheath as a potential biomaterial for assessment of neurotoxicity of endocrine disrupting chemicals

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Myelin sheath plays a crucial role in proper neuronal function by providing trophic and metabolic support to axons and facilitating energy-efficient saltatory conduction. The disruption in the myelin sheath leads to the lower or blocked nerve impulse transmission. Myelin sheath is affected by many internal (genetic factor) and environmental factors (endocrine disrupting chemicals – EDCs). To study the neurotoxicity of endocrine disruptors in terms of the myelin sheath, a zebrafish model was selected. It is an alternative and early developmental model and shares 70% of gene homology with humans. Several studies have documented the toxicity of EDC (di(2-ethylhexyl)phthalate) on the liver, heart, kidney, etc. However, there is a lack of knowledge regarding the toxic effects of this chemical on the myelin sheath of the brain. We have investigated the neurobehavioral, histopathological, and molecular changes in the larvae at 5 days post fertilization after being exposed to two different doses of DEHP (50 and 100 ppm). Cuprizone was taken as the positive control. The eye movement of the zebrafish was decreased with the increasing dose of DEHP. While there are no changes in motor movement. Histological data revealed an increased cell death in DEHP-treated groups. The expression of myelin sheath-related proteins (SOX10, MBP, and MAP2) was decreased in DEHP-exposed larvae. In summary, this study shows the toxicological potential of DEHP in affecting the myelin sheath during the developmental stages of zebrafish larvae which can be conveniently used for study of molecular mechanisms of neurotoxicity of environmental endocrine disruptors.

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Microphysiological systems as a tool to assess systemic toxicity of chemicals

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Systemic toxicity assessment is a gap in the safety evaluation of chemical ingredients, as there is no validated alternative to traditional animal testing, which are banned for cosmetics context due to human relevance and ethical reasons. Read across and threshold of toxicological concern are potential alternatives, but these rationales have some limitations as the absence of analogues for the first one and application for lower amounts and conservative maximum permitted exposure for TTC, which impacts directly in the assessment of the safe concentration of these ingredients.

Microphysiological systems (MPS) have emerged as promising alternatives to traditional animal testing for evaluating the systemic toxicity of cosmetic ingredients. These systems mimic the complex physiological environment of human tissues and organs, providing more predictive and ethical approaches to toxicity assessment. By integrating microfluidic technologies and human-derived cells, MPS enable the study of absorption, distribution, metabolism, and excretion (ADME) processes, as well as organ-specific toxic effects. Their application in cosmetics ingredients safety evaluation enhances compliance with animal testing bans while improving the reliability of toxicological predictions.

We combined a microphysiological system of three organ equivalents (full-thickness skin, liver and intestine) and transcriptomics to assess the systemic toxicological potential of chemical ingredients. The organ equivalents were in depth characterized, including morphological and functional features of each one. The systemic toxicity potential was assessed through the modulation of a gene panel comprising many toxicity biomarkers in liver and intestine equivalents, after dermal exposure. Acetaminophen and formaldehyde were selected as chemical-tests, since they are known systemic toxicants with published data to compare.

Our results showed this methodology has the potential to be applied in an integrated approach to assess systemic toxicity and other systemic outcomes, since many hepatotoxicity, intestinal toxicity and carcinogenicity gene markers were modulated after exposure, as expected for tested chemicals.

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Engineering 3D liver models in hydrogel

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Drug-induced liver injury (DILI) is a leading cause of drug failure in clinical trials and accounts for more than 50% of acute liver failure cases, mainly due to the low concordance between preclinical models and human liver toxicity. Although animals models and 2D cell cultures are widely used to assess the drug toxicity in preclinical trials, they have limitations in accurately predicting human responses. Therefore, there is an urgent need to develop physiologically relevant human-derived liver models for hepatotoxic screening. Three-dimensional (3D) models offer a more predictive approach as they better replicate the natural cellular microenvironment, including cell-cell interaction and extracellular matrix (ECM) dynamics.

In this study, we investigated three 3D hepatic models (HepaRG:HHSTec) to evaluate liver toxicity: (1) liver cells directly encapsulated and differentiated in hydrogel, (2) spheroids encapsulated within the hydrogel and (3) spheroids differentiated in absence of hydrogel. Spheroids were formed in an agarose mold, and all models were differentiated into mature hepatocytes in culture medium with 2% (v/v) DMSO for 14 days. The hydrogel was composed of GelMA (3%) and 4-arm-PEG (1%), mimicking the ECM.

Cell viability was assessed using Live/Dead and CellTiter-Glo® assays, confirming the hydrogel's biocompatibility. Additionally, a significant difference in spheroid diameters was observed among the models, with model 3 exhibiting a greater reduction in diameter compared to model 2, suggesting higher stability in the latter. Hepatic functionality was evaluated through biochemical assays (urea, albumin, glucose, ALT, and AST) and immunofluorescence, using differentiation markers (CYP3A4, MRP2 and CK-19).

These findings highlight the potential of hydrogel-based 3D hepatic models for improving DILI prediction. Further studies will refine these models and validate their ability to enhance preclinical drug screening accuracy, ultimately contributing to the development of more reliable alternatives to animal testing.

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Exploring vascularization strategies in collagen-based tissue constructs for advanced 3D tissue models

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Emerging regulations are restricting the use of animal models in line with ethical concerns. Furthermore, the inaccuracy of animal model predictions has been consistently identified, which emphasizes the need to develop alternative *in vitro* models that are reliable, enhance translational relevance, and correspond to the 3Rs approaches (refinement, reduction and replacement). In this context, 3D *in vitro* models have emerged as valuable tools for preliminary research, drug development, toxicology studies, and disease modeling, providing deeper insights into tissues pathophysiology. Vascularization is a key element in the development of these models, since it enables tissue perfusion and nutritional diffusion, impacting their physiological relevance and functionality. To construct vascularized 3D tissues, it is necessary to have an appropriate combination of extracellular matrix and a mixed cell population of endothelial and supportive cells, like mesenchymal stem cells (MSC) and fibroblasts. Hence, in the present study, different strategies of vascularization were explored in distinct tissue models, aiming to test the vascularization of collagen-based constructs to develop more robust and physiological *in vitro* models. The first proposed model was constructed by encapsulating fibroblasts (HDF) into a collagen matrix, and by adding the endothelial cells (HUVEC) to the surface of the construct. The structure of the tissue was then observed and evaluated through immunofluorescence, and it was evidenced that the endothelial cells stayed restricted to the surface, forming a well-organized and structured sheet. Also, an adipose tissue model was developed in which hMSC cells were encapsulated into collagen-based matrix, exposed to adipogenic differentiation induction protocol, and then, again, HUVEC cells were added to the construct surface. The immunofluorescence analysis revealed that the endothelial cells remained primarily on the surface, with some dispersed signals detected within the matrix, suggesting limited migration or initial organization. Then, another vascularization strategy was adopted and applied to a bi-layer skin model, with construction methodology already consolidated in the group. Now, the HDF and HUVEC cells were mixed and encapsulated into the collagen matrix in two proportions, 1:1 and 1:2, HDF and HUVEC respectively. Keratinocytes (HEKa) were then added to the construct surface to form the epidermis layer. The early observations of this third model suggested that the endothelial cells will undergo minimal self-organization, potentially forming early microvascular structures within the matrix. Overall, considering that the engineered tissues have different cellular composition, thus distinct differentiation and maturation processes, the results demonstrate the potential of collagen matrices as a platform for vascularized tissue modeling. Whereby, the vascularization strategy may vary depending on the target tissue and the particularities of the model being developed.

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Computational methods for the identification of bioactive peptides with therapeutic potential

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Therapeutic peptides (ranging from 2 to 50 amino acids) have emerged as promising candidates for disease treatment due to their high specificity, effectiveness, easy degradability, lack of toxic byproducts, and potential for reuse within the body. As a result, they are frequently utilized in medical practice, and their usage is anticipated to grow in the future.

Recent advancements in computational methods and the growing use of artificial intelligence have led to a revolution in various fields, including drug discovery. In computer-aided drug design (CADD), a range of techniques are employed to examine pharmacokinetic and pharmacological/pharmacodynamic properties. One such approach is Quantitative Structure-Activity Relationships (QSAR), which involves creating statistical models – often utilizing machine learning algorithms – to predict molecular properties. Furthermore, molecular modeling techniques, such as molecular docking, molecular dynamics simulations, and alanine scanning, play a crucial role in understanding molecular interactions and analyzing the stability of peptide-protein complexes.

In this study, we have developed different QSAR models to predict key properties of peptides, such as water solubility, plasmatic half-life, and hemolytic activity, to ensure the bioavailability and safety of peptides. These models have been implemented in a computational tool called Peptikinetics. Additionally, we have performed molecular docking to identify peptides capable of binding to the Pann1 channel and Cx43 hemichannel, thereby modulating their activity. We have complemented this study with molecular dynamics simulations to analyze possible structural changes in the proteins. Thus, in this work, we demonstrate the usefulness of computational approaches to identification of novel therapeutic peptides.

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Revealing the molecular language of kidney cells: How growth factors and cytokines shape iPSC-derived proximal tubular cells

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The kidney's proximal tubule is crucial for reabsorption, and detoxification, yet it remains highly vulnerable to injury and drug-induced toxicity. Traditional models – including animal studies, immortalized renal cell lines, and primary human proximal tubule cells – have advanced nephrotoxicity research but often lack full physiological relevance. Immortalized cell lines, despite their robust proliferative capacity, may exhibit altered gene expression and metabolic activity, while primary cells, although more physiologically accurate, are limited by donor variability and finite lifespan.

Induced pluripotent stem cell (iPSC)-derived proximal tubular-like (PTL) cells represent a promising human-specific alternative that better mimics the structure and function of renal proximal tubule cells. However, the molecular response of PTL cells to key growth factors and cytokines – critical mediators of kidney homeostasis, injury, and repair – remain unexplored. This study investigates the transcriptional changes in PTL cells after brief exposure to selective cytokines and growth factors, aiming to yield new insights into renal physiology, disease progression, and toxicological mechanisms.

Using a 14-day standardized differentiation protocol (Chandrasekaran et al., 2021), iPSCs were differentiated into PTL on Geltrex-coated plates and exposed to a cocktail of factors including IL-1 β , TGF- β 1, TNF- α , Activin A, BMP-4, EGF, FGF-1, HGF, and Wnt-3A, for 4 and 8 hours. TempOSeq transcriptomic profiling was then performed to identify gene expression changes.

We hypothesize that short-term exposure of iPSC-derived PTL cells to selected growth factors and cytokines will trigger rapid transcriptional changes, particularly in genes governing inflammation, epithelial repair, fibrosis, and metabolic adaptation. We expect these changes to mirror native renal responses, thereby reinforcing the potential of PTL cells as a robust, human-specific, animal-free model for nephrotoxicity screening and kidney disease research. Moreover, the insights gained from these studies may lead to improvements in the PTL differentiation protocol by optimizing growth factor application. Ultimately, this model may enhance our understanding of early kidney injury mechanisms and improve preclinical testing strategies.

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Advancing cell-based assay reproducibility through lens-free imaging and AI integration

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The 3R (Replacement, Reduction, Refinement) principle is a cornerstone of ethical and innovative advancements in life science research, particularly in the transition toward microphysiological systems (MPS) and away from traditional animal models. While significant progress has been made in Refinement, achieving effective Replacement and Reduction remains challenging due to the limited availability of robust alternative methodologies. Conventional cell-based assays, often integral to MPS, face critical limitations such as poor reproducibility, dependency on cell quality, and a lack of real-time, temporal information.

To overcome these barriers, we introduce an innovative approach for non-invasive, incubator-compatible monitoring of cell-based assays that aligns with the 3R principles. Leveraging lens-free imaging, our method captures the inherent optical properties of cells to enable label-free, continuous observation within a standard CO₂ incubator. Furthermore, real-time data processing is integrated with artificial intelligence (AI) algorithms, enabling the automated analysis of critical parameters such as confluency, proliferation, and motility [1].

This study highlights the utility of our system through experiments on two colon carcinoma cell lines, cultured under arsenic trioxide treatment, where significant memory effects from subculturing influenced growth patterns and multi-omics profiles. Notably, higher growth homogeneity in early passages correlated with an 80% increase in differentially expressed markers [2].

Our system offers substantial advantages for MPS applications, including label-free quantification of organoid growth dynamics and 3D cell interactions, supporting the Replacement of animal models. By improving assay reproducibility, data quality, and standardization, this approach represents a transformative step toward addressing the reproducibility crisis in life sciences and advancing the adoption of MPS technologies.

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Advancing *in vitro* approaches for medical device safety assessment: Special irritation protocols

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Introduction: Medical device safety evaluation relies on robust biocompatibility assessments to ensure compliance with international regulatory frameworks. The “Big Three” testing concept – cytotoxicity, irritation, and sensitization – forms the foundation of ISO 10993 guidelines. While standardized *in vitro* methods for cytotoxicity are well established, challenges remain in irritation and sensitization assessments, particularly for ocular and oral exposure routes. Our recent work addresses these gaps by advancing protocol development and validation efforts for irritation testing in reconstructed human tissue models.

Methods: Building on our latest publications and ISO10993-23 protocol for intracutaneous skin irritation testing, we have optimized and pre-validated *in vitro* approaches for ocular and oral irritation testing, utilizing reconstructed human cornea-like and oral mucosal models (EpiOcular and EpiOral). In addition to irritation, we also addressed photo-irritation reactions that may occur in dentistry or during exaggerated exposure to sunlight, further expanding the applicability of *in vitro* models in medical device safety assessment. The standard irritation protocol involved 21 ± 1 hours of exposure, followed by a cytotoxicity test (MTT assay) and IL-1 α analysis. The photo-irritation protocol was extended by one additional day, incorporating an irradiation step followed by a post-incubation period, as outlined in OECD Test Guideline 498. The test materials included extracts from medical devices (MDs) as well as over-the-counter (OTC) products, ensuring broad applicability to various consumer and healthcare-related products.

Results: Our findings corresponded well with the known effects of studied Medical device materials and products. The research demonstrated the applicability of advanced *in vitro* models for special irritation testing, offering reproducible and mechanistically relevant outcomes. The optimized protocols align with regulatory expectations and provide a promising pathway for reducing reliance on animal testing in medical device evaluation. Furthermore, our work contributes to the broader integration of non-animal methods into ISO 10993 frameworks, paving the way for improved safety assessments.

This research has been supported by APVV grant APVV-19-0591 In vitro biocompatibility testing of medical devices and new-generation biomaterials

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Unveiling the dynamics of scratch assays: Continuous, label-free monitoring of cell migration using lens-free imaging and AI

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Scratch assays are essential for studying cell migration and wound healing in cellular biology. Traditional methods, relying on intermittent measurements and labeling, can disrupt cellular behavior. This study introduces a novel approach utilizing continuous, automated, lens-free imaging and analysis within CO₂ incubators, providing detailed and accurate analysis of scratch assay dynamics.

We developed an advanced system for non-invasive, label-free imaging to continuously monitor the closure of scratch “wounds” in 2D cell cultures. This methodology enables real-time data acquisition, significantly enhancing the temporal resolution of cell movement observations compared to manual acquisition (every 30 minutes vs. every 8-12 hours). By tracking closure speed and direct cell movements without external markers, we gain a comprehensive understanding of cell dynamics.

Our findings demonstrate that this continuous monitoring system not only accurately measures wound closure rates but also distinguishes between active directed migration and proliferation-driven movements. This distinction is critical, as it provides a reliable indicator for differentiating these mechanisms in a label-free environment. The ability to discern these processes opens new avenues for understanding cell behavior in various biological contexts, including cancer metastasis and tissue regeneration.

Overall, this study underscores the potential of automated, continuous lens-free imaging systems in CO₂ incubators to revolutionize cell migration assay analysis. By eliminating disruptive labeling and enhancing data resolution, our approach significantly advances cellular dynamics research, providing nuanced insights into cell behavior and movement mechanisms.

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Development of a diabetic 3D skin model

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3D bioprinting has emerged as a promising technique for creating non-clinical human models, providing a new platform to study diseases such as Diabetes mellitus (DM). Impaired wound healing is a major challenge associated with DM-related metabolic complications. Moreover, animal models used to study these mechanisms are limited by high costs and low predictive accuracy, adding complexity to the investigation of these conditions. This study aims to develop and validate a 3D diabetic skin model for *in vitro* wound healing assays. To construct a robust model, we first optimized a three-layered skin structure by evaluating different extracellular matrix compositions. Next, we selected the most effective model for inducing a diabetic skin phenotype. The model construction involves bioprinting a hypodermal layer consisting of human mesenchymal stem cells differentiated into adipocytes and embedded in a collagen-based bioink. A dermal layer, composed of human fibroblasts in a collagen matrix, is then bioprinted over the hypodermis, followed by the addition of a stratified epidermal layer using primary human keratinocytes. Variations in the dermal matrix were introduced by formulating different hydrogels, combining type I collagen with other proteins such as elastin, fibronectin, and additional biomolecules. Cell viability analysis in the human skin equivalent with hypodermis (HSEH) model showed a reduction in ATP levels on day 10 of differentiation, suggesting process stabilization and lower metabolic activity. Morphological evaluation revealed a homogeneous distribution of fibroblasts in the dermis and a stratified epidermis, indicating an organized structure. Additionally, complex human skin equivalent (HSE) models and scaffold-based models exhibited improved fibroblast distribution and a more structured dermis, suggesting greater physiological viability. After selecting the most physiologically relevant model, we will induce a diabetic skin phenotype using various biochemical cocktails to modulate the skin's physiological conditions in the next phase of our project.

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Mechanoregulation of synovial fibroblasts: Unraveling fibrotic activation and ECM remodeling in osteoarthritis using a microfluidic chip

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Background: Osteoarthritis (OA) affects approximately 7% of the world's population and is characterized by joint degeneration, chronic pain, and inflammation, often worsened by mechanical stress. Fibrosis, a hallmark of OA, involves excessive fibroblast activation induced by chronic tissue injury, mechanical stress, and low-grade inflammation. While mechanical stimuli are crucial for joint homeostasis, they also play a central role in cell activation, extracellular matrix (ECM) remodeling, and disease progression. The underlying mechanisms linking mechanical stress to OA progression are crucial for the development of effective treatments but are still elusive. However, conventional models often rely on animal models, highlighting the need for physiologically relevant, animal-free alternatives.

Objective: This study aimed to establish a fully animal-free *in vitro* model of the synovial membrane using a microfluidic chip to investigate the impact of mechanical compression on synovial fibroblast activation, ECM remodeling, and mechanosensitive signaling pathways relevant to OA progression.

Methods: Primary human synovial fibroblasts from OA patients were embedded in a fibrin matrix and cultured for seven days on a microfluidic chip (BiomimX) under controlled mechanical compression (0%, 10%, and 30%). RNA sequencing, histological, and biochemical analyses were performed to assess fibrotic activation and ECM remodeling.

Results: Mechanical compression induced fibrotic activation and ECM remodeling, contributing to OA progression. RNA sequencing identified 12,027 commonly regulated genes, with distinct gene expression patterns at different compression levels. Gene Ontology analysis revealed that 10% compression triggered the unfolded protein response, while 30% compression enhanced collagen catabolic processes and ECM organization. In addition, histological analyses demonstrated increased ECM deposition, α -smooth muscle actin expression, and matrix metalloproteinase production under 30% compression compared to 0%, indicating fibrotic activation and enhanced ECM remodeling.

Conclusion: This study introduces a human-based microfluidic model that highlights the crucial role of mechanical stress in modulating ECM remodeling and cellular stress responses in OA. By identifying distinct molecular pathways involved in fibrotic activation and ECM dynamics, this model provides a robust and (patho)physiologically relevant platform for studying the mechanobiological properties of joint tissues offering deeper insights into OA pathogenesis.

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NAMs for safety assessment of novel botanical ingredients in cosmetics: A case study

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Innovation in the cosmetics sector is linked to the development of new ingredients capable of promoting differentiated benefits to finished products. A differential is the use of ingredients from Brazilian biodiversity, since the search for formulations that have new bioactives with high performance has increased significantly in the market. The safety assessment of these botanical ingredients presents a series of challenges, as they are complex mixtures of natural products. These molecules, belonging to different chemical classes, often have unprecedented molecular structures never before evaluated and, therefore, without toxicological data available in the literature. Given the need to generate these data through New Approach Methodologies (NAMs), an integrated strategy of different methods in a weight-of-evidence approach is necessary for the conclusion on each toxicological outcome.

In this work, a case study on the safety assessment of a new botanical ingredient, developed from Brazilian biodiversity, will be presented. For this assessment, different *in silico* models and *in vitro* methodologies were combined, covering several toxicological outcomes, in addition to exposure calculation rationales, for the conclusion on the safe use concentration of the new ingredient in different product categories.

The characterization of the phytochemical composition of the ingredient for the identification of the main molecular markers was conducted using robust analytical techniques. Subsequently, an in-depth literature search was carried out in the main toxicological databases and relevant scientific articles. For natural products without available data, *in silico* rationales were applied to generate data on different toxicological outcomes. In these analyses, QSAR Toolbox, VEGA, Pred-skin, Toxtree models and a proprietary artificial intelligence model were applied.

For the *in vitro* methodologies carried out with the botanical ingredient, Ames (OECD 471) and micronucleus (OECD 487) tests were performed to assess the mutagenic and genotoxic potentials, respectively, in addition to an internally developed methodology (SenScreen) for the assessment of dermal sensitization, combined with a clinical compatibility test (HRIPT).

For the assessment of the systemic toxicity of molecules without data available in the literature, the Threshold of Toxicological Concern (TTC) rationale was applied considering the class of concern according to the chemical structure of each natural product. Finally, safe concentrations of the botanical ingredient were established for application in different product categories.

Thus, it is verified that the use of new approach methodologies for the safety assessment of natural ingredients proved to be an effective strategy, allowing the use of innovative ingredients that impact product performance, and ensuring consumer safety in a robust way.

Presentation: Poster

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Assessment of cytotoxicity, skin, and oral mucosa irritation of medical devices used in the oral cavity: A case study

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Introduction: Ensuring the safety and biocompatibility of medical devices (MDs) is critical to protecting patient health and complying with regulatory requirements. *In vitro* safety testing plays a key role in identifying potential risks, reducing reliance on animal testing, and ensuring the suitability of MDs for human use. This study aimed to evaluate the *in vitro* cytotoxicity and irritation potential of the medical device “Hydro Pastilky” following ISO 10993 guidelines. A benchmark product with a similar composition and mode of action was included for comparative assessment.

Methods: For cytotoxicity evaluation, the Neutral Red Uptake (NRU) test (Annex A, ISO 10993-5) was used. Cell cultures treated with “Hydro Pastilky” exhibited viability above 50% up to a 34.5% neat extract concentration, with significant decreases observed at higher concentrations based on microscopic analysis of cell morphology, density, and viability. The benchmark product showed unnatural OD increases at the two highest concentrations, likely due to Neutral Red dye binding to its partially jellified extract. The cytotoxic effects at higher concentrations were attributed to the water-binding capacity of the products, which may alter cell osmolarity over prolonged exposure.

For irritation assessment, the EpiDerm and EpiOral reconstructed tissue models were employed following ISO 10993-23. Extracts (0.2 g/mL) from “Hydro Pastilky” and the benchmark product consistently resulted in viabilities above 50%, with most exceeding 75%, classifying both products as non-irritating. The responses in both tissue models were highly comparable, further supporting their safety profile.

Conclusion: Both “Hydro Pastilky” and the benchmark product demonstrated no significant skin or oral irritation effects, confirming their biocompatibility under normal conditions of use. These findings support their classification as non-irritating medical devices for oral cavity applications.

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Presentation: Poster



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The impact of social isolation on laboratory rodent health, replicability and translatability: A systematic review and meta-analysis

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Over 120 million rodents are used in biomedical studies globally each year, yet most of this research is non-replicable and very little translates to human therapies (termed the replicability and translatability “crises”, respectively). Furthermore, many laboratory rodents are isolated in barren, “shoebox-sized” conventional housing that compromises welfare, inducing chronic stress. Our previous meta-analysis showed that, compared to rodents in larger, well-resourced enclosures allowing the expression of natural behavior, those in small conventional cages are more susceptible to stress-sensitive diseases (anxiety, cancer, cardiovascular disease, depression, stroke) and have shortened lifespans. Additional meta-analyses of interactions between housing (conventional versus well-resourced) and disease modifiers being studied (e.g., therapeutic drugs) revealed that housing quality can alter research findings, impacting replicability. Furthermore, using conventional housing may overestimate the therapeutic benefits of tested drugs, impacting translatability. Here we build on this work to investigate chronic stress effects in individually housed rodents. We hypothesize that poor welfare from social isolation similarly impacts animal health. We further hypothesize that these effects similarly alter research findings, impacting replicability and translatability. To test these hypotheses, we conducted a systematic review. Following best practices for reducing bias, we detailed our research plan in an online protocol prior to conducting our study (<https://hdl.handle.net/10214/28126>). We searched five databases (Ovid, CABI, Web of Science, Proquest, SCOPUS) on May 26, 2024, finding 2,384 potentially relevant articles. Two independent reviewers conducted all steps to minimize error and bias. We screened all articles for eligibility (published in English, using mice or rats, and using social isolation as an intervention), resulting in 121 articles meeting inclusion criteria from which data were extracted. These spanned all five previously studied stress sensitive diseases, and mortality, while including one new disease: viral infection. Twenty-eight articles also assessed the efficacy of disease modifiers. Data analysis is ongoing, via random effects meta-analyses, including meta-analyses of statistical interactions (using “article” as the unit of replication). Results will help reveal how social isolation impacts laboratory rodents’ health and address whether these effects can alter experimental results, reducing replicability and translatability. These data, combined with previous work on rodent housing, will allow us to assess the importance of welfare as a key variable that should be considered when conducting animal research, and when addressing both replicability and translatability crises. If our hypotheses are supported, this would suggest that social housing is important not just for animal welfare but also for rigorous, effective biomedical science.

Presentation: Poster

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Monkey business: Trends in importation and use of *Macaca fascicularis* in Canada

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Background: Non-human primates (NHPs) are still considered to be the best substitute for humans in biomedical research and regulatory testing, despite mounting evidence proving their failure to recapitulate human responses. Cynomolgus macaques (*Macaca fascicularis*) are the most commonly used NHP in science. In their indigenous home of South and Southeast Asia, facilities breed them for exportation and use in science worldwide. This leads to these highly intelligent and social animals accounting for 10% of the annual revenue of all animals exported globally. Until 2019, China was the largest supplier of purpose-bred macaques, but they reduced their export by 96% in 2019, compared to average of 2016-2018; other countries, particularly Cambodia, increased their export to meet the demand. In 2022, following the IUCN uplisting of these macaques to endangered, US federal prosecutors charged 2 Cambodian officials and 6 employees of a primate supply organization with passing off wild macaques as captive-bred, resulting in a suspension of trade of macaques between Cambodia and the US. Given the close trade relationship between US and Canada, we examined trade databases to determine the impact of these events on the trade and use of these macaques in Canada.

Methods: We used the CITES trade database, Statistics Canada’s Canadian International Merchandise Trade Web Application, and the Canadian Council on Animal Care Annual Animal Use (CCAC) Data Reports to determine changes in patterns of Canadian import, export, and use of cynomolgus macaques and their specimens.

Results: Following the de facto trade ban between Cambodia and US, there was a 447% increase in import of primates into Canada from Cambodia between 2022 and 2023 (StatsCan) with more than 99.7% and 98% of NHPs imported into Canada in 2022 and 2023, respectively, being cynomolgus macaques (CITES). Commensurate with this increase in importation, the CCAC reported an increase in the use of macaques from 7651 macaques being used in CCAC-certified labs in 2022 to 11,991 macaques in 2023 – the highest ever use of macaques in Canada. Disconcertingly, we also identified a recent trend of importation of wild macaques into Canada (CITES); 732 wild macaques in 2021, 645 in 2022, and 399 (incomplete data) in 2023. A similar trend was also seen with wild macaque sourced specimens.

Conclusions: Importation and use of wild macaques is problematic due to the physiological and psychological trauma to the animals themselves, the risk of zoonotic diseases, and the unreliability of data acquired using wild animals. Canadian import and use of cynomolgus macaques have increased drastically since 2022 and we discovered a troubling trend of importation of at least 1796 wild macaques into Canada between 2021-2023 (potentially incomplete data). This finding will be used to lobby for legislative and regulatory changes that would ban the import of all wild non-human primates to into Canada. The sharp increase in importation and use of macaques in research and testing in



Canada over the past few years, despite their inability to recapitulate human physiology, will be used to campaign funding bodies to improve funding to the develop and validate more humane and human-centric non-animal alternatives. Swift and decisive changes to both trade laws and funding priorities are required to ensure the survival of this endangered species.

Presentation: Poster

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Engineering bioinks for organotypic *in vitro* models

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Developing physiologically relevant human *in vitro* models is essential for improving the predictive power of non-clinical testing systems and reducing reliance on animal models. Tissue engineering has focused on creating organotypic *in vitro* models using human cells and advanced technologies such as 3D bioprinting. A key challenge in bioprinting is selecting an optimal bioink composition tailored to each cell type and tissue structure. The bioink formulation must not only support the printing process but also preserve cell viability and functionality, ensuring the proper assembly of tissue-specific architectures. Here, different bioinks based on distinct combinations of gelatin methacryloyl (GelMA), fibronectin, elastin, laminin, extracellular matrix (ECM), platelet-rich plasma (PRP), and plasma rich in growth factors (PRGF) were

evaluated for their biocompatibility with different cell types, focusing on their potential application in 3D culture models. GelMA provides a photocrosslinkable bioink that enables the printing of 3D models, while the inclusion of biological components enhances cell functions. For biocompatibility evaluation, cells were encapsulated in the hydrogels (10,000 cells/50 μ L) and, after crosslinking, maintained in culture for up to 30 days. Cell morphology and growth capacity were analyzed by fluorescence microscopy, while cell viability was indirectly assessed by ATP quantification or directly evaluated using a live/dead assay. To explore the vascularization potential of 3D bioink models, GelMA 4% containing 10% PRP was used to encapsulate human endothelial cells (HUVECs) for testing. For the development of parenchymal tissues, human mesenchymal stem cells (hMSCs) were encapsulated in GelMA 4% formulated with either 50% ECM (2); FEL – fibronectin (40 μ g/mL), elastin (9 mg/mL), and laminin (20 μ g/mL) (3); or 10% PRFC (4). Overall, all bioinks exhibited biocompatibility and promoted cell proliferation. Due to their epithelial-like profile, HUVECs did not grow well inside the hydrogels. However, when cultured on the surface, they proliferated successfully, demonstrating the hydrogel's potential for printing vessel walls. All biomolecules tested in combination with GelMA supported hMSC proliferation, with ECM and PRFC inducing more robust cell growth compared to the FEL composition, likely due to their more complex and bioactive nature. Nevertheless, FEL provided a more homogeneous and controlled composition, which could be advantageous for bioink manufacturing and the construction of 3D models. The results demonstrated the compatibility of different biomolecules, creating a versatile portfolio that allows for the selection of bioink components tailored to the specific needs of each organ. The ability to fabricate constructs with customizable geometry using bioactive inks containing cells holds significant promise in advancing 3D culture models, providing flexibility and optimizing functionality for specific applications.

Presentation: Poster



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Unraveling the role of osteopontin-c in B-cell acute lymphoblastic leukemia cell invasion: Insights from a blood brain barrier-on-a-chip model

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Background: The blood-brain barrier (BBB) is a highly selective interface that regulates molecular exchange between the bloodstream and the brain. Understanding how B-cell acute lymphoblastic leukemia (B-ALL) cells infiltrate the central nervous system (CNS) is crucial for improving treatment strategies and enhancing our understanding of the underlying mechanisms of leukemia cell transmigration. Our group previously identified osteopontin-c (OPN-c) as being highly expressed in B-ALL patients with CNS infiltration, and its expression correlates with poor prognosis.

Aims: This study aimed to develop a BBB-on-a-chip model that closely mimics the physiological architecture of the human BBB microenvironment to investigate the mechanisms by which OPN-c may modulate B-ALL CNS invasion.

Methods: We engineered a microfluidic BBB-on-a-chip platform that incorporated two cell layers – one containing human brain endothelial cells (hCMEC/D3) and the other composed of astrocytes and pericytes – separated by a porous membrane. The system was continuously perfused with culture media at a controlled flow rate of 10 μ L/h. Barrier selectivity was characterized by perfusing fluorescent dextran

molecules with molecular weights ranging from 40 to 150 kDa, and the leakage score was calculated. Immunofluorescence assays were used to analyze the expression of proteins at the BBB cell-cell tight junctions. The RS4;11 B-ALL cells were either silenced or not silenced for OPN-c using anti-OPN-c or scrambled DNA oligomers and then stained with CellTracker Deep Red and Green dyes. These cells were perfused into the BBB-on-a-chip system for 12 hours. Cell images were acquired using confocal microscopy and image analysis was performed using Fiji/ImageJ. The B-ALL cells perfused into the chip were analyzed across approximately 200 Z-stacked images, which were captured in between the layers of the BBB-on-a-chip and summarized into 9 slices and 8 sub-slices containing the BBB cells.

Results: The BBB-on-a-chip system displayed permeability scores that were twice as restrictive for molecules ranging from 40 to 150 kDa, indicating robust barrier selectivity. We also observed homogeneous fluorescence intensity distribution of tight junction proteins within the hCMEC/D3 monolayer. 3D imaging and orthogonal analysis confirmed strong intercellular connectivity at the BBB and B-ALL cell transmigration through the BBB microfluidic system. Both RS4;11 cells with silenced and non-silenced-OPN-c cells showed a progressive decrease in cell number along the chip slices where the BBB was positioned. Notably, 20% more OPN-c silenced cells were found at the BBB cell region compared to control cells, which also displayed earlier and later modifications in their circularity index, both before and after crossing the slices in which the BBB cells were positioned. These findings suggest that OPN-c silenced cells are retained at higher levels or adhere more efficiently to the BBB cells than control cells with higher OPN-c expression.

Conclusions: Our engineered BBB-on-a-chip provides a functional and physiologically relevant model for studying leukemic cell invasion into the CNS, demonstrating that higher levels of OPN-c may facilitate CNS invasion through the BBB. Furthermore, this platform serves as a powerful tool for dissecting the molecular mechanisms underlying CNS B-ALL transmigration, further emphasizing the role of OPN-c in B-ALL progression.

Presentation: Poster



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ToxiGut: Modeling and prediction of drug and chemical side effects in an *in vitro* 3D reconstituted human small intestine model

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Introduction: Assessing drug and chemical toxicity in the gastrointestinal (GI) system is critical for ensuring human safety. However, due to physiological differences, traditional animal models often fail to predict human responses accurately. ToxiGut aims to bridge this gap by developing a 3D reconstituted human small intestine model integrated with a microfluidic device to evaluate intestinal toxicity and absorption of pharmaceuticals and chemical substances.

Methods: A 3D *in vitro* model of the small intestinal epithelium (Epi-Intestinal) was used in the experiments and integrated into a dynamic microfluidic bioreactor to better mimic physiological conditions. In parallel, the CaCO₂ cell line was used as a model broadly used by the pharmaceutical industry. The model evaluated the cytotoxicity and absorption of three selected chemicals, employing markers of barrier integrity (TEER), inflammation (Cytokine release), and cytotoxicity.

Results: The first data indicate that the “ToxiGut” concept replicates intestinal absorption patterns observed in human physiology. Toxicity assays demonstrated the ability to differentiate between safe and harmful substances, with key findings supporting the role of fluid dynamics in modulating barrier function and cellular responses. Comparative studies with static *in vitro* systems revealed improved predictability and higher sensitivity in detecting adverse effects in the ToxiGut microfluidic model.

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Presentation: Poster

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Neurotoxicity assessment of consumer product chemicals using machine learning and *C. elegans* models

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With the increasing application of artificial intelligence (AI) in toxicology, efforts are underway to integrate big data such as high-throughput screening (HTS) results into chemical risk assessment. Traditional experimental methods are not suitable for large-scale chemical screening, highlighting the need for computational approaches. The Adverse Outcome Pathway (AOP) framework provides a mechanistic bridge between *in vitro* HTS data and *in vivo* toxicity endpoints, facilitating explainable predictions. In this study, we developed a knowledge-based AI model guided by neurotoxicity-related AOPs for screening the neurotoxicity of consumer product chemicals and to validate the predictive outcomes using *Caenorhabditis elegans* (*C. elegans*) behavioral assays. Key events (KEs) from established neurotoxicity AOPs were mapped to relevant ToxCast *in vitro* assays, and *in vivo* toxicity data were curated from various open-source databases. Machine learning models – including Random Forest, XGBoost, Gradient Boosting, Decision Tree, and Logistic Regression – were built using molecular fingerprinting methods (MACCS, Morgan, Layered, RDKit). The models were used to screen a wide range of chemicals present in consumer product databases. Among the predicted neurotoxicants, we selected high-risk chemicals for experimental validation using *C. elegans* locomotive behavior assays. The *in vivo* validation supported the accuracy of our predictive model. This study demonstrates a mechanistically informed AI framework that enhances model explainability through integration of *in vitro* and *in vivo* data. Our approach offers a proactive solution for early-stage screening of neurotoxicity, enabling prioritization of chemical risks prior to regulatory assessment.

Presentation: Poster



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Self-assembling peptide hydrogels as user-defined synthetic matrices for *in vitro* modelling

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Introduction: Current 3D *in vitro* models of development and disease typically rely on animal-derived materials to represent the matrix. These materials suffer from an ill-defined composition, high batch-to-batch variability, and do not accurately reflect human biology. This is a major contributing factor to the high attrition rate of new drugs moving from the lab to the clinic, which is particularly high for new anti-cancer therapies. This has driven the recent interest in developing more reliable, synthetic alternatives which allow for customization of the mechanical and biological properties of the matrix to better reflect human biology.

Materials and methods: Using a simple self-assembling peptide hydrogel (SAPH), we have developed an innovative, and animal-free, 3D cell culture platform to address the current shortcomings of available *in vitro* models. The SAPH is well defined, has limited batch variability, and can be customized to mimic the mechanical and biological features of specific tissues more faithfully when compared to traditional animal-derived materials.

Results: We have demonstrated the platforms usefulness in modelling a range of different tissue types, including several different types of cancer. This includes breast cancer, glioblastoma, colorectal cancer, hepatocarcinoma, and acute myeloid leukemia, for both mechanistic studies and drug screening. Each tissue type requires specific tuning of the mechanical and biological features of the SAPH to promote the viability and correct *in vivo*-like behaviors of encapsulated cells.

Conclusion: Use of a chemically-defined, and animal-free, 3D cell culture platform can potentially replace the use of materials like Matrigel®, but more work is needed to elucidate the right combination of features (mechanics and composition) relevant to each cell line and application.

Presentation: Poster

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SARA-ICE: A self-contained model for predicting a human relevant point-of-departure for skin sensitization

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Skin Sensitization Risk Assessment – Integrated Chemical Environment (SARA-ICE) is a Bayesian statistical model that estimates a human-relevant metric of skin sensitizer potency. This metric, termed ED01, is the dose with a 1% chance of human skin sensitization following a human predictive patch test (HPPT) exposure. SARA-ICE accounts for input data variability and explicitly quantifies uncertainty in estimating the ED01. Possible input data includes HPPT (human repeat insult patch test or human maximization test), *in vivo* local lymph node assay (LLNA), and new approach methods (NAM [*in chemico* direct peptide reactivity assay (DPRA), kinetic DPRA and *in vitro* KeratinoSens™, h-CLAT, or U-SENS™]).

Two versions of SARA-ICE have been developed. One, the SARA-ICE defined approach (DA), is under evaluation by the OECD for inclusion in Test Guideline 497, Defined Approaches for Skin Sensitisation, with Guideline updates underway. This DA allows derivation of a point-of-departure (POD), defined as the mean of the ED01 given the assumption the material of interest is a sensitizer. The SARA-ICE PoD (estimated using a limited set of NAM inputs) was compared to various sets of reference values, including Local Lymph Node Assay (LLNA) median-like location parameter (MLLP) and a weight of evidence dose per skin area which sensitizes 1 test subject (DSA1+). For all sets of comparisons, absolute bias between the SARA-ICE DA POD and the reference value is approximately 2-fold. Predictive performance of the mean PoD against reference DSA1+ is comparable to the predictive performance of the LLNA MLLP.

Further to the SARA-ICE DA, a SARA-ICE “Extended” Model is shared which allows derivation of UN Globally Harmonized System of Classification and Labelling of Chemicals (GHS) hazard and potency categorizations. In addition, the SARA-ICE Extended Model also allows users to visualize the full distribution of the ED01, accounting for parameters such as variability in the input assays.

The models do not require a specific dataset to make predictions, thus allowing for iterative and flexible application based on available data, which is demonstrated through application of a case study. Recently, a web application has been developed, allowing local installation and making SARA-ICE DA and extended versions available to all stakeholders. This presentation will highlight the differences in the models, their evaluation and application.

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Presentation: Poster



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Comparison of human equivalent doses derived from regulatory data and new approach methodologies for per- and polyfluoroalkyl substances-related liver toxicity

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While many legacy poly- and perfluoroalkyl substances (PFAS) are now regulated, a wide range of alternative PFAS compounds continue to be used. Next generation risk assessment (NGRA) methods have been proposed as efficient tools to replace traditional risk assessment for these emerging alternatives. This study aimed to compare PFAS liver toxicity data derived from regulatory and new approach methodologies (NAMs), based on an adverse outcome pathway (AOP) network linking liver steatosis to PFAS regulatory endpoints. Twelve target chemicals were selected for this study, including four regulated PFAS, three restricted PFAS, and five alternative PFAS compounds. First, we reviewed international risk assessment reports to identify liver toxicity endpoints supporting PFAS regulation. These regulatory endpoints were then integrated into an AOP network associated with liver steatosis, a well-known PFAS-induced disease, using resources such as the AOP-Wiki, the Comparative Toxicogenomics Database (CTD), and expert knowledge. Next, NAMs data were systematically collected from peer-reviewed literatures and ToxCast, a high-throughput screening database, and classified according to relevant toxicity events. Finally, the collected *in vitro* data were divided by C_{ss} (steady-state plasma concentration) of each chemical to calculate human equivalent doses (HED). *In vivo* data from regulatory reports were converted to HED using allometric scaling factor. This study evaluated the applicability of NAMs-based hazard assessment methods compared to existing PFAS regulatory values. Our findings propose an NGRA strategy based on available toxicity data to support regulatory decision-making. Additionally, this research contributes to narrowing the gap between scientific research and regulatory, providing a comprehensive overview of the current state of PFAS liver toxicity knowledge.

This research was supported by a grant (24192MFDS076) from Ministry of Food and Drug Safety in 2025.

Presentation: Poster

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Integrating *in silico*, *in vitro*, and *in vivo* data to identify developmental neurotoxicity of plastic additives chemicals

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As widely used plastics break down into smaller particles in the environment, exposure to additives in plastics can lead to toxic effects on human health and ecosystem through various pathways. There is growing evidence that exposure to plastic additives potentially affect development and behavior. However, existing *in vivo* testing methods are inadequate for evaluating developmental neurotoxicity (DNT) for the plastic additives lacking toxicity data. In this context, integrated assessment combining *in silico*, *in vitro*, and *in vivo* data was conducted to provide insight into the of DNT of plastic additives.

First, we employed molecular docking to predict binding affinities between plastic additives and receptors implicated in DNT. Among the screened additives, Bisphenol A (BPA) exhibited the strongest predicted binding affinity. Subsequently, bioactivity data from ToxCast assays relevant to the DNT-*In vitro* Battery (DNT-IVB) were utilized to train machine learning models, including Random Forest, XGBoost, Gradient Boosting, Decision Tree, and Logistic Regression, based on molecular fingerprinting techniques (MACCS, Morgan, Layered, RDKit). Notably, BPA and Di(2-ethylhexyl) phthalate (DEHP) showed significant bioactivity in these prediction models.

To further substantiate the evidence for DNT, additional *in vitro* and *in vivo* experiments were conducted using human embryonic stem cells, human neural stem cells, and wild-type and mutant strains of *Caenorhabditis elegans* (nlg-1(lf) and nrx-1(lf), mutants associated with synaptogenesis). Exposure to BPA and DEHP led to significant alterations in locomotor behavior in *C. elegans*, and levels of BDNF in human stem cells were also notably affected. Integrative evaluation across ToxCast assays, *in silico* molecular docking and machine learning predictions, stem cell-based *in vitro* assessments, and *C. elegans in vivo* studies collectively identified BPA as the chemical with the highest DNT potential.

Our study presents an integrating ToxCast data, computational modeling, *in vitro* stem cell assays, and *in vivo* nematode models, offering a robust approach to comprehensively screen the DNT potential of chemicals.

Presentation: Poster



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Integrating *in silico*, *in vitro*, and *in vivo* data for the genotoxicity and carcinogenicity of per- and polyfluoroalkyl substances

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Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants with potential genotoxic and carcinogenic effects, raising concerns about their impact on human health. While epidemiological studies suggest an association between PFAS exposure and increased cancer risk, a systematic approach integrating predictive modeling with experimental validation is required for hazard characterization. This study applies an Integrated Approach to Testing and Assessment (IATA) by combining *in silico* modeling, *in vitro* assays, and *in vivo* data to evaluate PFAS-induced genotoxicity and carcinogenicity.

In silico models, including molecular docking and quantitative structure-activity relationship (QSAR) analysis, were used to predict DNA damage potential and tumorigenic pathways. These predictions were validated through *in vitro* experiments, including Comet assay for DNA strand breaks, p53 quantification for tumor suppressor regulation, and cell transformation assay (CTA) to assess oncogenic potential. Additionally, *in vivo* studies were analyzed to establish dose-response relationships and confirm mechanistic insights.

Our findings indicate that *in silico* predictions align with experimental results, demonstrating PFAS-induced oxidative stress, DNA repair inhibition, and dysregulation of tumor suppressor pathways. Comet assay confirmed significant DNA damage, while p53 quantification and CTA supported the potential for PFAS-mediated carcinogenic transformation. These results highlight the utility of IATA in integrating computational and experimental approaches for a comprehensive assessment of PFAS-related health risks.

This study underscores the importance of mechanism-based hazard characterization and supports the application of IATA frameworks in regulatory toxicology for PFAS risk assessment.

Presentation: Poster

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Integrating heart-on-chip technology and 3D porcine living myocardial slices for cardiotoxicity screening

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Introduction: Cardiotoxic events are major concerns in pharmaceutical drug development and a leading cause for market withdrawal. One of the key cardiotoxic drivers is the interaction of compounds with ion channels (e.g., human ether-à-go-go (hERG) K⁺ and L-type Ca²⁺ channel), affecting cardiomyocyte action potentials and potentially triggering life-threatening arrhythmias. The prevailing preclinical testing strategies rely on the use of cell lines (e.g., transfected HEK293, CHO) that express hERG artificially or iPSC-derived cardiomyocytes. However, these models lack the structural and functional complexity when compared to 3D mature myocardium and intrinsic cardiomyocyte action potentials. To circumvent these experimental limitations, we validated a microphysiological system (MPS) for 3D *ex vivo* living myocardial slice (LMS) cultivation with an integrated dual-pacing strategy for direct assessment of cardiac action potential duration.

Methods: MPS was applied by combining left ventricular porcine LMS (300 µm) on a heart-on-chip approach, which allows advanced nutrition circulation and integrates electrical pacing for cardiomyocyte contraction. Slices were paced using a dual (S1S2) pacing protocol to monitor action potential duration in functional cardiac tissue. Contractile activity was detected through motion vector-based video analysis. Contractility parameters were analyzed after applying pro-arrhythmic drugs to porcine LMS.

Results: Porcine LMS were successfully generated and could be electromechanically stimulated in MPS culture conditions. Action potential duration was recorded after dual pacing and revealed similar durations compared to human hearts. In line, proof-of-concept study with the specific hERG channel blocker dofetilide confirmed prolongation of action potential duration of 3D myocardium in a dose-dependent manner. Functional effects were monitored within minutes after drug application to the model system. LMS were also treated with cisapride, which was originally developed for reflux and heartburn but was withdrawn earlier from the market due to cardiotoxic events. Of great interest, cisapride resulted in concentration-dependent lengthening of LMS action potential duration underlining the pre-clinical potential of such drug testing platform.

Conclusion: The combination of MPS with electrically paced, *ex vivo* 3D LMS provides a physiologically relevant platform for cardiotoxicity testing. Our heart-on-chip model enhances translatability compared to traditional 2D cell culture models by integrating mature porcine cardiac tissue, which expresses a comprehensive set of ion channels crucial for action potential formation and thereby is an ideal mimic of the human heart. We also postulate multi-dose compound testing from a single donor heart, supporting drug safety evaluation while reducing animal studies. The herein described approach can be universally applied in pre-clinical drug development to screen for potential cardiac off-target effects.

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Advancing the 3Rs at a medical faculty: Pioneering projects in replacement, reduction, and refinement

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The responsible implementation of the 3Rs (Replacement, Reduction, and Refinement) is crucial for ethical and high-quality biomedical research. Charité – Universitätsmedizin Berlin, a leading institution in translational medicine, is driving three lighthouse projects within Charité 3R that significantly advance the 3Rs principles.

Replacement: Turning Clinical Waste into Scientific Gold – The Primary Tissue Pipeline (PTP): In many clinical procedures, valuable human tissue is discarded despite its potential to revolutionize biomedical research. The Primary Tissue Pipeline (PTP) addresses this gap by establishing a service platform that facilitates access to living human bioprobes for research. This initiative enhances the use of organoid models and direct tissue cultures, promoting personalized medicine and regenerative therapies. By bridging the gap between healthcare and research, the PTP provides ethically sourced, high-quality tissue samples, accelerating scientific discovery while reducing reliance on animal models.

Reduction: EPIC3R – Experimental Imaging at Charité Platform: Pre-clinical imaging plays a pivotal role in reducing the number of animals required for research while increasing the scientific value of studies. EPIC3R (Experimental Imaging at Charité) integrates multimodal imaging technologies to refine experimental designs, enabling longitudinal studies that minimize animal use. By offering a centralized infrastructure for small animal imaging, including, e.g., MRI, PET, CT, and intravital microscopy, EPIC3R supports translational research and contributes to digital *in silico* modeling. This initiative fosters innovative approaches that optimize preclinical studies and enhance clinical translation.

Refinement: Refinement Sharepoint – A Digital Platform for Enhanced Animal Welfare: Refinement strategies aim to improve animal welfare and experimental procedures. The Refinement Sharepoint platform at Charité provides a collaborative, in-house knowledge-sharing system for researchers, veterinarians, and technicians. This internal platform offers protocols, instructional videos, and expert contacts to facilitate best practices in humane animal handling and procedural refinement. By fostering a protected and supportive environment, the Refinement Sharepoint ensures continuous improvement in animal welfare while maintaining scientific rigor.

These initiatives underscore Charité's commitment to responsible research and the advancement of the 3Rs. By implementing sustainable structures that enhance replacement, reduction, and refinement, these projects contribute to ethical, innovative, and high-impact biomedical research.

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PFAS mixtures exhibit additive effects in a hiPSCs-based embryotoxicity model

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Humans are exposed to many chemicals through diet, environment and personal care products. Among these, there are per- and polyfluoroalkyl substances (PFAS), a class of highly persistent substances which have been associated with adverse developmental effects in humans. In the present study, we assessed the effect of four PFAS (PFOS, PFOA, PFNA and PFHxS) and mixtures thereof in the PluriLum assay, a 3D hiPSCs-based tool for embryotoxicity testing.

Firstly, we evaluated the effect of the single PFAS on cardiomyocyte differentiation, showing that PFNA was the most potent substance followed by PFOS, PFOA and PFHxS. Subsequently, three reconstituted mixtures of these four PFAS were tested. The mixtures were prepared either at equipotent levels (at their benchmark concentration of 10% of differentiation inhibition, or BMC10; “balanced mixture”) or at average serum levels reported for the population of adult or children in Europe (“real-life mixtures”). The comparative assessment between observed and predicted mixture responses demonstrated concentration additivity across all tested concentration ranges. The reconstituted real-life mixtures indicated a large safety margin for the populations in terms of embryotoxicity, with effects in the PluriLum assay seen above an enrichment factor of 600.

Finally, RNA sequencing analysis revealed that PFOS, PFOA and PFNA, at their BMC10 level, affected functional pathways related to cardiac/cardiomyocyte/muscle tissue development, as well as genes associated with extracellular matrix, focal adhesion and cell-substrate junction deregulation. Only two protein-coding genes were significantly deregulated by all three PFAS, which were related to cardiac development and membrane transport activity. PFHxS induced non-significant transcriptome changes. Overall, PFOS had the largest impact on pathways related to embryonic development.

In conclusion, the PluriLum assay can be used to detect PFAS embryotoxicity. We demonstrated that mixtures of four PFAS followed the principle of concentration addition, supported by shared functional pathways, indicating common sites of molecular action.

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Impact of losartan on molecular pathways related to renal injury: An *in silico* approach to inflammatory responses and cellular stress

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Potassium losartan is widely used in Brazil for the treatment of hypertension and heart failure. However, its off-label use in patients with kidney disease raises concerns regarding its safety, highlighting a gap in the understanding of the molecular mechanisms leading to nephrotoxicity. Indeed, drug safety is crucial for human health, and in this context, innovative methodologies, such as New Approach Methodologies (NAMs), particularly computational ones, have emerged as promising alternatives to enhance the evaluation of toxicity mechanisms and adverse effects while also contributing to the reduction of animal models in various stages of research and drug development. This study aimed to evaluate, through an *in silico* approach, the impact of losartan on key molecular pathways associated with renal injury, focusing on the modulation of inflammatory responses and cellular stress. The methodology involved data mining from the Comparative Toxicogenomics Database (CTD) and GeneCards, using the keywords “losartan,” “kidney disease,” “gene,” and “inflammation.” Biological pathways were mapped and analyzed using the Reactome platform and were selected based on their modulation in relation to the search terms. To expand the comparative perspective, data from known nephrotoxic drugs, such as gentamicin, cyclosporine, and doxorubicin, were included, allowing for the identification of common pathways. Enrichment analysis revealed a predominance of critical pathways, including interleukin signaling, immune responses, cellular stimulus responses, and cellular senescence, with high statistical significance ($p < 0.0001$; $FDR < 1 \times 10^{-10}$), indicating a relevant impact on renal homeostasis. The computational approach adopted refines and directs hypotheses regarding toxicity mechanisms, highlighting the potential of NAMs for preliminary drug safety assessments. These findings provide a solid foundation for future studies, which should include *in vitro* assays and molecular biology investigations to validate the proposed mechanisms and guide clinical strategies for therapeutic safety.

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The role of *in silico* methods in predictive toxicology

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Predictive toxicology is an important area of study in contemporary toxicology that encompasses *in silico*, *in chemico* and *in vitro* approaches, highlighting the concept of New Methodological Approaches (NAMs) in the quest to predict toxicological effects of compounds. The products generated from these toxicological approaches are sources that feed computational models, aiming to predict risks associated with xenobiotics and promote the development of new biomarkers. Additionally, predictive toxicology plays a key role in improving the drug development process enabling early screening and prioritization of molecules, while also presenting great potential in the regulatory context. The objective of this study was to investigate *in silico* tools in predictive toxicology and their applications. The results obtained demonstrated that several computational techniques are employed in this field, some of which are machine learning, quantitative structure-activity relationship models and molecular docking. Machine learning has driven several advances in computational chemistry. It is an analytical method that automates the construction of analytical models using algorithms that iteratively learn from data. In the context of predictive toxicology, it is applied in the molecular properties prediction, pharmacokinetic and pharmacogenomic modeling, chemical synthesis prediction and identification of biomarkers. Quantitative structure-activity relationship (QSAR) and quantitative structure-property relationships (QSPR) models are powerful computational modeling tools used for determining relationships between the chemical structures of molecules and their biological activities. They are widely applied to predict the toxicological effects of chemicals on human, animal and environmental health. The integration of QSAR/QSPR approaches with machine learning techniques enhances the development of more robust models. Another key *in silico* approach in predictive toxicology is molecular docking, a technique that identifies the preferred orientation of a given molecule in relation to a target macromolecule, while scoring the strength of the binding interactions, predicting the binding affinity of molecules in the active site of a given receptor, also being used in the verification of xenobiotic interactions in relation to biological targets. It is a potential tool for regulatory purposes, being successfully applied in the development of classification models for the prediction of the endocrine disrupting potential of some chemicals. Overall, predictive toxicology is an essential field of study for the rational development of drugs and implementation of alternative methods to animal testing. Through the use of computational approaches in the study of the toxicological potential of compounds, it is possible to achieve greater efficiency in relation to classical methods. However, it is necessary to study the limitations that each approach may present. This study emphasizes that the application of *in silico* techniques in elucidating toxicity mechanisms represents a powerful tool for risk characterization and regulatory application, constituting a fundamental component of predictive toxicology.

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Colorectal cancer organoid-on-a-chip for drug sensitivity testing

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The development of advanced *in vitro* models has been critical in improving cancer drug screening. Widely used traditional 2D cell cultures have many limitations, such as the absence of tumor heterogeneity, lack of tumor microenvironment complexity, and lack of 3D architecture. Here, we propose that the most promising approachable models are cancer organoid-on-a-chip models, which combine 3D cancer organoids with microfluidic devices. These systems provide the tumor microenvironment and organ-level physiological responses, providing a more accurate and controlled environment for drug testing. The fluidic microenvironment allows for dynamic, real-time drug exposure to tumor cells, more accurately reflecting physiological drug exposure in human patients.

Colorectal cancer is now the third leading cause of cancer-related death in women and men, mainly because of the high incidence of cancer metastasis and chemotherapeutic treatment inefficiency. Therefore, *in vitro* cancer models are important to evaluate the efficacy of compounds, identify drug resistance mechanisms, and explore therapeutic targets. Cancer organoid models more closely mimic the structure of tumors *in vivo*, including cell-cell and cell-matrix interactions, and allow for the creation of drug-resistance niches.

ATCC provides over 300 next-generation cancer models, manufactured, characterized, and validated in-house, available for academic and commercial use to aid in cancer research and drug discovery, including 78 expanded colorectal cancer organoids. These models are provided through the Human Cancer Models Initiative (HMCI).

In this study, we demonstrate that colorectal cancer organoid-on-a-chip are used for drug sensitivity test to deliver compounds to *in vitro* cancer models in the real physiological drug exposure. Data suggest that novel tumoroid-on-a-chip microfluidic system mimics the real microenvironment of a specific tumor to the complex organ-level physiological and pharmacological responses.

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ASPIS academy: Early-stage researchers in next-generation toxicology

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The ASPIS Academy is a dynamic networking and training platform designed to support the professional development of early-stage researchers (ESRs) within the ASPIS cluster, which unites three European Horizon 2020 projects – ONTOX, RISK-HUNT3R, and PrecisionTox. Established in 2023, the ASPIS Academy is committed to advancing the next generation of toxicologists by providing tailored training programs, mentorship, lab exchange opportunities, and professional networking initiatives.

With a membership of over 120 ESRs, the ASPIS Academy plays a pivotal role in accelerating the transition towards animal-free chemical risk assessment by fostering expertise in new approach methodologies (NAMs). These cutting-edge approaches leverage *in vitro* and *in silico* models, as well as emerging technologies such as artificial intelligence (AI), omics, and advanced computational modeling. By equipping young researchers with essential scientific and transferable skills, the ASPIS Academy ensures they are well-prepared to lead innovation in the field of toxicology.

Led by ESR representatives and guided by experienced researchers, the ASPIS Academy cultivates an inclusive and collaborative environment where diverse perspectives thrive. Through its initiatives, the ASPIS Academy is shaping the future of chemical safety assessment in the European Union, driving scientific excellence, and contributing to a sustainable transition away from traditional animal models.

Visit our poster to learn more about our key initiatives, achievements from the past year, and exciting plans for the future. Discover how the ASPIS Academy is creating opportunities for early-stage researchers and how you can get involved!

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Development of an organ-on-a-chip model to simulate gut-liver bypass

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The development of innovative *in vitro* models that accurately replicate human physiology is crucial for reducing reliance on animal methodologies and improving the translatability of preclinical studies. This study presents the development of a gut-liver organ-on-a-chip (OoC) model designed to simulate the metabolic interactions between the gastrointestinal tract and the liver. This system aims to provide a physiologically relevant platform for evaluating the bioavailability and metabolic transformation of pharmacological drugs and nutraceuticals.

The gut-liver OoC model integrates a microfluidic device housing co-cultures of intestinal epithelial cells and hepatocytes. The model enables dynamic flow conditions that mimic the physiological environment, including nutrient absorption, first-pass metabolism, and hepatic biotransformation. By replicating these key processes, the system allows for an in-depth assessment of how bioactive compounds are processed, metabolized, and made available to systemic circulation. The model is under development using a combination of microchip and tissue engineering techniques. The design of the model is debated by analyzing different options available in the literature. One model was chosen to evaluate cells physiology and connection with microfluidic. Different parameters are being studied to understand the ideal combination. The next steps include validation of a reference compound to compare the results with known *in vivo* metabolic pathways.

This model represents a step forward in preclinical research by enhancing the understanding of human-specific drug metabolism while aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement). The gut-liver OoC has significant potential for application in pharmaceutical and nutraceutical research, supporting the development of safer and more effective compounds tailored to human physiology.

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Advances and trends in phototoxicity testing: A review of the scientific literature

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The development of alternative methods to animal experimentation has gained prominence in assessing the toxic potential of compounds exposed to sunlight, especially in the cosmetics and pharmaceutical sectors. The phototoxicity tests are harmonized by OECD, existing today 3 test methodologies, and one integrated approach to testing and assessment. Considering that validated methods exhibit robust scientific publications before they are accepted by the OECD, this study carried out a preliminary review of recent scientific literature to identify available knowledge trends and advances in the field. Thus, the databases PubMed, Scopus, Google Scholar, and Scielo were consulted, using the following keywords and Boolean operators “Phototoxicity test” OR “*in vitro* phototoxicity” OR “Phototoxicity assay” OR “Phototoxic potential” OR “Photo-irritation” OR “photobiology” AND “OECD Test Guideline” OR “3T3 Neutral Red Uptake assay”. Only articles and reviews from 2004 to 2024 were selected. Of the 14 documents found, 9 were selected after reading the abstracts. The results highlight the importance of reactive oxygen species (ROS) generation as a central mechanism of phototoxicity, with studies using ROS assays to assess the photoreactivity of pharmaceutical and cosmetic products. In addition, progress has been made in the international harmonization of testing strategies, with the adoption of guidelines such as OECD TG 495 and ICH S10. Comparative studies between different biological systems, such as cell lines (Balb 3T3) and stratified keratinocytes, have demonstrated the effectiveness of *in vitro* methods in assessing phototoxicity. *In silico* tools, such as Derek Nexus and QSAR Toolbox, have also been evaluated for their ability to predict the phototoxicity of chemical substances, showing promise for reducing the use of animals in tests. The application of phototoxicity testing has been extended to new contexts, such as the evaluation of tattoo pigments and the detection of phototoxic contaminants such as benzo[a]pyrene. In addition, the development of reconstructed human epidermis models, such as KeraSkin™, has represented a significant advance in the validation of alternative methods. Studies on natural compounds, such as phenolic antioxidants and furocoumarins, have also contributed to understanding the mechanisms of phototoxicity and the safety of cosmetic ingredients. Recent advances in the field of phototoxicity have been driven by the integration of *in vitro*, *in silico*, and *in chemico* methods, with a focus on reducing animal experimentation and improving test accuracy. As we can see, the search in the scientific literature reveals new methods/models that should be incorporated into the OECD and reveals applications of existing methodologies in new products, such as tattoo inks.

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Structuring of a microfluidic system (AdipoChip) and evaluation of its predictive power as an alternative tool to animal use in preclinical drug toxicity assays

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Introduction: Animal models are commonly used in pharmaceutical research to predict human drug toxicity. However, they present limitations due to interspecies differences, variability among test subjects, and ethical concerns regarding animal stress and suffering. Consequently, alternative methodologies are essential to improve toxicological assessments while reducing reliance on animal testing. Organ-on-a-Chip (OOC) technology has emerged as a promising approach, integrating human cells and microfluidic systems (MFS) to mimic physiological processes better.

Objective: This study aimed to standardize an adipose tissue OOC model, named AdipoChip, as an innovative tool for toxicological risk assessment.

Methods: Human adipose-derived stem cells (hASCs) commercially purchased (Cat. Num. PT-5006, Lonza®, Walkersville, USA), (Ethics Committee approval CAAE: 10917112.9.0000.5248) were cultured in four-channel chips connected to infusion pumps, generating a continuous flow of 10 µL/h and in 96-well plate. The cells were maintained for 10 days in a culture medium containing adipogenic differentiation inducers, and different doses of the test substance for cytotoxicity evaluation. At the end of the experiment, cells were fixed and stained with Nile red to label lipid vesicles and DAPI to identify nuclei. Morphological analysis was performed using the High-Content Screening system (Operetta CLS, PerkinElmer) and Harmony 4.9® software.

Results: hASCs were able to differentiate better into adipocytes in microphysiological systems (MPS) compared to static 96-well plate cultures. Adipogenic differentiation was enhanced in AdipoChip, as evidenced by a greater lipid droplet area in differentiated cells compared to conventional culture. A flow rate of 10 µL/h was optimal for uniform differentiation, avoiding the gradient effect observed at higher flow rates. It was possible to establish dose-response curve, based on the four-slope hill function, based on the inhibition of adipogenesis in MPS and the correct toxicity category was predicted for the tested substances, in 10 days, in comparison to the 14 days' assays previously described.

Conclusion: The AdipoChip platform successfully reproduces key physiological aspects of adipogenic differentiation under dynamic conditions, demonstrating its potential as a reliable tool for toxicological screening and reducing the need for animal testing.

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Establishing a standardized Brazilian RhCE model for ocular irritation testing: A transition to animal-free methodologies

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The human corneal epithelium is highly susceptible to damage from chemicals and environmental factors, reinforcing the need for reliable ocular irritation assessment models. Traditional methods relied on animal testing, but ethical concerns and scientific advancements have driven the development of alternative approaches, such as reconstituted human corneal epithelium (RhCE) models. Despite the existence of well-established international models, the absence of a standardized national model in Brazil limits broader implementation. This study aimed to establish and validate a reproducible, accessible RhCE model based on the ToxIn Ocular protocol. The primary goals were to employ a chemically defined, animal-free culture medium and address limitations in existing methods, promoting ethical and scientifically robust toxicological evaluations. For that, HaCaT keratinocytes were cultured on 24-well inserts coated with bovine collagen I at a density of 5×10^5 cells/insert. Four culture media (A, B, C, and D) were assessed for tissue morphology and functionality, with medium D selected for further testing. The model's effectiveness in ocular irritation assessment was evaluated using 15 substances from the OECD Test Guideline No. 492 proficiency list. Additionally, HaCaT cells were adapted to an animal-free environment by replacing fetal bovine serum (FBS) with human serum (HS) in the culture medium, trypsin, and freezing medium. Morphological characteristics, gene expression (MKI67, CDH1, CK14), and cell doubling time were analyzed. No significant differences were observed in tissue morphology and functionality across tested media conditions. The selected protocol effectively distinguished non-classified substances from irritants, confirming its potential for ocular irritation testing. The transition of HaCaT cells to an animal-free culture system, particularly with 5% HS supplementation, did not compromise key cellular properties. This study successfully refined the ToxIn Ocular model, significantly reducing reliance on animal-derived components. The developed protocol enables accurate irritancy assessments, demonstrating the model's potential for identifying non-irritating substances. Furthermore, transitioning HaCaT cells to an animal-free environment maintained cellular integrity, supporting the ethical and scientific shift away from animal testing. Developing a national model could facilitate regulation, reduce dependence on imported methods, and enhance accessibility for research and industrial applications, reinforcing the need for standardized, reproducible methodologies in toxicological assessments.

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Using the largest number of tools available in surgical education and training, reliable results are obtained

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The use of laboratory animals has been the gold standard for training in disciplines such as surgery, being a controversial issue that is not limited to the scientific field, having legal implications, conscientious objection and impact on the media and public opinion. The objective is to demonstrate how training and education methods have been modified over the years in a Public Pediatric Hospital to achieve acceptable levels of learning.

Through alternatives such as: use of inanimate material, simulation, software, reuse of cadavers, tissue preservation and 3D printing, a permanent training program was developed.

By comparing learning results generated by the alternative methods, additional benefits were obtained such as time and cost savings, improved potential for parameterization and repetition of the learning exercise, increased surgeon confidence and satisfaction, greater compliance with legislation on the use of animals, elimination of objections to the use of animals sacrificed for specific purposes and the integration of clinical perspective and ethics in the early stages of training of surgeons.

Evidence shows that educators can better serve their students, while minimizing financial and time burdens, with the introduction of well-designed training methods that do not depend on the harmful use of animals.

The gradual implementation of low-cost and high-tech models make a perfect match in the tools necessary to carry out such Training and Capacity Building Task.

In recent years we have witnessed great scientific advances, one of them being the development of 3D printing in different fields, including cardiovascular surgery, whose fundamental role has been the planning of surgeries to repair congenital cardiovascular defects and in endovascular procedures: coarctation of the aorta, hypoplastic arch and double outflow tract of the right ventricle, among others, which allow the reconstruction of three-dimensional images of cardiac pathologies through augmented reality, virtual reality and 3D printing, and are used in treatment planning.

The experience of both the surgeon and the bioengineers is limited and requires constant feedback between the surgeon's surgical needs and the alternatives that the bioengineer can offer between structures: transparent and hollow materials to see internal structures and instrument them; opaque and hollow materials for the reconstruction of large vessels and cardiac chambers; and finally, opaque and solid as a recreation of hard tissues such as calcifications, thrombi or tumors.

The work of an interdisciplinary group of different professionals who can analyze the same information through different methods is very important.

We are therefore at a turning point. Science and Society are advancing compared to a few years ago, but there are still many points to cover and many obstacles to overcome. It is therefore important that the scientific community continue to make efforts to support alternative methods, to continue investing in their development and implementation in all areas, since in this way we will all benefit: humans, because we will develop more reliable models, and non-human animals, because we will avoid unnecessary suffering.

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Replacing fetal bovine serum with platelet-rich plasma in the isolation of extracellular vesicles from adipose tissue-derived mesenchymal stromal cells

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The use of fetal bovine serum (FBS) in cell culture is controversial due to ethical and scientific reasons. The way it is obtained, through cardiac puncture of bovine fetuses without anesthesia, as well as the high variation between batches and the restrictions for its application in clinical contexts are some reasons that lead to the development of alternatives for its use in primary research. Platelet-rich plasma (PRP), a platelet-concentrate in a small volume of plasma, is a possible alternative to SFB, with promising results in terms of its ability to promote cell proliferation even in smaller concentrations. A particular interest in the substitution of SFB for PRP is in mesenchymal stromal cell (MSC) culture, given their therapeutic potential for proliferation and regeneration that has been directly linked to the production of extracellular vesicles (EVs). Studies show that PRP may be able to enhance this potential, however more investigation is needed. MSC-derived EVs have been tested as possible therapeutic agents in the Regenerative Medicine field given their ability to carry bioactive molecules from the MSC to the target cells, which allows them to modify their phenotype or functional behavior. Evidence also shows that EVs originating from MSCs perform functions analogous to those of the MSCs themselves, with adipose tissue-derived MSCs indicated as having a high regenerative potential for cartilage and bone. Thus, the goal with this project is to substitute SFB with PRP in the supplementation of culture media for adipose tissue-derived MSCs and to evaluate the effects in the synthesis of EVs. EVs were isolated from the supernatant of adipose tissue mesenchymal stromal cell cultures ($n = 3$) by ultracentrifugation (Type 70 Ti Fixed-Angle Rotor) and characterized for size and concentration using the Nanoparticle Tracking Analysis (NTA) technique and for morphology using Transmission Electron Microscopy (TEM). The NTA technique indicated that it was feasible to isolate similar amounts of extracellular vesicles from the culture supernatant of adipose tissue MSC when supplemented with 10% fetal bovine serum (FBS) compared to supplementation with 2.5% PRP ($p > 0.05$). In addition, we found that the isolated vesicles were similar in size in both scenarios (SFB: 127.5 ± 3.2 nm, 125.2 ± 10.9 nm, 150.6 ± 1.8 nm; PRP: 113.7 ± 6.4 nm, 190.5 ± 2.5 nm, 162.5 ± 1.6 nm; $p > 0.05$). Transmission Electron Microscopy revealed no significant morphological changes between the EVs of the groups supplemented with PRP and SFB. Additional analyses, including qPCR, for expression of miRNAs modified during osteoarthritis (miR-140 and miR-337), and Western Blot, for expression of typical vesicle proteins (CD63, CD81 and TSG101), are in progress. From the results obtained so far, we can conclude that it is possible to replace the use of SFB, a xenogenic product, with PRP, of human origin, to obtain extracellular vesicles from human adipose tissue-derived MSCs.

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Regulatory acceptance of alternative methods to the use of laboratory animals in safety studies of products for human use and agrochemicals in Latin America

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The use and acceptance of alternative methods to animal testing in regulatory studies of products for human use and agrochemicals in Latin America is still limited. Currently, the legislation for the registration of these products and the regulatory authorities responsible for their authorizations require toxicological studies carried out in animals and the acceptance of studies carried out by alternative methods is limited. In this context, we will review the current methods available, the different technologies under development in the short, medium or long term that would allow us to replace, reduce or refine the current toxicological methods. Additionally, we would review the current legislation in Latin America regarding the toxicological evaluation of products and their acceptance by the regulatory authorities. This review is a useful tool to exchange knowledge, disseminate progress and experiences of the different countries and promote the acceptance of alternatives to animal testing in toxicological studies of products in Latin America. This information is of great interest to the representatives of the industry, academy and government, in order to develop different policies and promote regulatory changes in their countries and regions.

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EU-approved monkey business for scientific purposes

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Non-human primates were assigned the role of our closest relatives, which translated to granting them special protection, but also to using them in biomedical research and testing. This apparent contradiction fuels turbulent ethical debates whenever authorities decide over experimentation proposals involving primates. However, what is not part of this consideration between estimated suffering and hoped-for scientific findings is the origin and sourcing of these animals.

5,784 non-human primates have been used for scientific purposes in 2022 in the European Union, 86% of these were long-tailed macaques (*Macaca fascicularis*). 98% of these macaques were born outside of Europe and then imported from Africa and Asia [1]. In the same year the long-tailed macaque was elevated to “endangered” on the International Union for Conservation of Nature Red List of Threatened Species [2], and it has long been known that the legal and illegal capture, breeding and trade of these macaques threatens human, animal and environmental health. Recent investigations unveiled sales of wild-caught macaques from Cambodia to the United States by illegally labelling them as captive-bred, amongst other cases involving countries with native macaque habitats – all indicators of an obscure global industry network [3].

We wanted to know how the EU is entangled in this web of primate trade and focused our research on Germany and the long-tailed macaque. The presentation will summarize a) the status quo and developments over recent years of trade and use of long-tailed macaques for scientific purposes, b) results from a stakeholder survey in Germany including authorities, companies, and research institutions to determine the level of awareness, knowledge and transparency, c) the current animals in science legislation in the EU to discuss whether it is sufficient to counteract the problem. Despite the high relevance on a scientific, ethical and legal scale these issues have been mostly tolerated. The purpose of this presentation is to keep a spotlight on them and voice a call for action and collaboration. What is the real cost of using primates for scientific purposes? And what is the real benefit of animal-free science?

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Wisdom begins in wonder – Igniting curious young minds with 3Rs science

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In our current society, many questions arise with respect to the use of laboratory animals in scientific research and their relevance to human health and our environment. Conversely, public trust in alternative methods remains low, partly due to unfamiliarity. By implementing early onwards education focusing on the 3Rs (Replacement, Reduction, Refinement), we aim to bridge the gap between society and scientists, ensuring that individuals from all societal levels understand the 3Rs and gain more confidence in alternative methods. As the Belgian Innovation Centre for 3Rs (IC-3Rs), it is our responsibility to correctly inform the public, spread the message as widely as possible, and help to create a better educated and ethical society regarding scientific practices and animal welfare, as such preventing polarization and misunderstanding.

Given the key role of synergy between science and education, we introduced 3R-educational material for schoolchildren between 11 to 14 years old. Various schools and educational institutes across Flanders and the Brussels Region were visited, educational material was made available, and teachers were guided through its practical use. Education in applying 3Rs fits both basic science classes (e.g., Biology) and philosophy lessons (e.g., Life classes), as it addresses not only the moral aspects of animal experimentation, but also explains the scientific mechanistic principles behind alternative methods. Also, other forms of science communication for children are being explored. For instance, IC-3Rs participates in various fairs where science is presented to and by youngsters. Hands-on workshops are being organized that allow children to investigate drug safety without animal testing. Discussions with young, yet inquisitive, “scientists” are interesting and enrich teachers as well as the audience.

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Galleria mellonella larva as a promising alternative model for investigation of paracoccidioidomycosis: Pathogen-host interaction and immune response to monoclonal antibodies against *Paracoccidioides lutzii*

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Introduction: *Galleria mellonella* larvae serve as a promising alternative model to mammalian studies due to their similar innate immune system, aligning with the 3Rs principles (Replacement, Reduction, and Refinement) in animal research. This model is particularly useful for investigating the immune response to pathogens such as fungi of the *Paracoccidioides* genus, agents of Paracoccidioidomycosis, an endemic mycosis in South and Latin America, primarily *P. brasiliensis* and *P. lutzii*. The production of monoclonal antibodies (mAbs), based on the classical technique by Köhler and Milstein (1975), combined with Multiple Tolerization Subtractive Immunization (MTSI) adapted by Neves et al. (2024), are used to enhance the understanding of immune response and facilitate the diagnosis of *P. lutzii*. These approaches have the potential to produce novel monoclonal antibodies against this pathogen.

Objective: Investigate antibody monoclonality and the protective effects of MTSI-produced anti-*Paracoccidioides lutzii* mAbs in *Galleria mellonella* larvae.

Methodology: For mAb production, the anti-*Paracoccidioides lutzii* hybridoma clone PL01S5C92-H10, developed by Neves et al. (2024) using MTSI, were cultured in RPMI medium with additives and 10% of fetal bovine serum (FBS). After confirming viability, FBS was gradually reduced to 1%, and mAb production was induced by medium acidification over 14 days (batch method). The supernatant was purified using an FPLC system with an IgG column, and SDS-Page electrophoresis confirmed the presence of antibodies. The reactivity of the mAb to exoantigens of *P. lutzii* (PL01 strain) was assessed by Western blotting. To evaluate the survival of *G. mellonella* infected with *P. lutzii* and *P. brasiliensis*, strains Pb18 (*P. brasiliensis*) and PL01 were cultured in YPD medium agar (Yeast Extract Peptone Dextrose) and adjusted to 6×10^6 cells/mL. Five groups of 10 larvae were incubated at 37°C: (1) unmanipulated; (2) injected with 10 µL of PBS; (3) injected with commercial IgG (10 µg/mL); (4) infected with Pb18 incubated with and without mAb (H10); and (5) infected with PL01 incubated with and without mAb (H10). Survival, cocoon formation, mobility, and melanization were monitored daily for 7-10 days to generate the health score and survival curves.

Results: The H10 hybridoma maintained adequate viability with low FBS concentrations, revealing a 150 kDa band under non-reducing conditions, and light (25 kDa) and heavy chains (50 kDa) under reducing conditions. Western blotting confirmed the reactivity of the H10 mAb to PL01 strain of *P. lutzii*, with a 50 kDa band, not previously described.



Larvae infected with the PL01 strain and incubated with the H10 mAb survived for 10 days, 5 days longer than the infected larvae without the antibody, as confirmed by the health score. This prolonged survival suggests that the H10 antibody exerts an immunoprotected effect.

Conclusion: *Galleria mellonella* larvae are a promising alternative model for investigating the fungus-host interaction and immune response to the monoclonal antibody H10, which exhibited high reactivity to *P. lutzii*. This model not only enhances the understanding of the paracoccidioidomycosis immunopathogenesis but also supports the reduction of traditional animal use in research, in line with the 3Rs principles, which are fundamental to ethical animal research.

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Institutional integration of 3Rs: A collective effort, not a one man show

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The implementation of the 3Rs (Replacement, Reduction, and Refinement) in research is a collective effort that extends beyond individual responsibility. In response to the Flemish Government's Action Plan to Reduce Animal Experimentation, the "Vrije Universiteit Brussel" established an interdisciplinary 3Rs working group, consisting of multiple laboratory directors and led by its Innovation Centre 3Rs (IC-3Rs). As such a university-wide strategy embedded within the broader Action Plan was developed. Collaborations were set up with other Universities and Research Centres to realize the overarching goal of minimizing the use of experimental animals.

The working group acts as a catalyst for various initiatives. Knowledge sharing, expanding the RE-Place project to centralize knowledge on non-animal methods in a national database and connecting with other researchers are the pillars of the Action Plan.

Education and practical skills training are realized through specialized hands-on workshops for PhD students, post-doctoral scientists and technical laboratory staff. Topics addressed so far consisted of "Severity scoring, Colony management, Culture of care, and Experimental design". By complementing these workshops with focussed webinars and expert-led discussions, a culture of ethical responsibility is actively established.

One of the most significant outcomes of the efforts made is a 10% reduction in the use of experimental animals over the past year, along with a 15% shift in classification of animal experiments from severe to mild. This positive trend is largely due to the alignment of severity scoring across the different universities in Flanders, demonstrating the impact of coordinated efforts.

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Gradual reduction of fetal bovine serum in cell cultures: Challenges and advances

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The use of *in vitro* tests without animal-derived reagents is a growing necessity in scientific research to ensure ethical and sustainable laboratory practices. One of the main challenges in this transition is the reduction or complete elimination of fetal bovine serum (FBS) from cell culture media, as it plays a crucial role in cell proliferation and maintenance. This study aimed to evaluate the feasibility of gradually reducing FBS in the culture of HaCaT keratinocytes and primary fibroblasts to determine their adaptability and proliferation capacity under serum-free conditions. A gradual reduction of FBS was performed on HaCaT cells and fibroblasts, starting from 10% FBS and decreasing to 7.5%, 5%, 2.5%, 1%, and finally 0%, with changes occurring every three days. A high initial cell confluence was used to mitigate the effects of reduced proliferation. Proliferation assays were conducted to assess cellular viability and adaptation. HaCaT cells successfully adapted to 0% FBS after 15 days, continuing to grow with a normal morphology. A proliferation assay at this stage revealed 70% confluence in the 0% FBS condition compared to 100% confluence in the 10% FBS control, indicating a decrease in proliferation but maintaining healthy cellular morphology. However, fibroblasts did not exhibit the same adaptability. Upon reaching 0% FBS, they showed signs of senescence, ceased proliferation, and eventually died. This study demonstrates the feasibility of serum-free conditions for HaCaT cells while highlighting the challenges faced by fibroblasts, emphasizing the need for further investigation into alternative culture media and supplementation strategies. Beyond FBS reduction, other animal-free substitutes are essential, including enzyme-free dissociation methods, such as non-animal-derived trypsin alternatives, serum-free cryopreservation media, and defined growth factors. Moreover, gene expression analysis techniques can be employed to validate cellular maintenance and adaptation in serum-free conditions. The continued development of serum-free methodologies is crucial for the advancement of ethical and reproducible *in vitro* models.

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Towards One Health: Navigating the current challenges of environmental safety of cosmetics

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Integrating environmental safety considerations into education and training is essential for promoting sustainable and responsible practices across sectors. At the Vrije Universiteit of Brussel, besides the yearly courses on “Safety assessment of cosmetics in the EU”, a new course titled “Introduction to environmental safety of cosmetics” was launched last year with support from leading European cosmetic companies. This course targets safety assessors to gain a general understanding of the complexities of environmental risk assessment. Focus is on regulatory challenges and opportunities for developing and integrating non-animal methods. In Europe, legislations are sector-specific, with different legislations for chemicals, pharmaceuticals, food additives and cosmetics. Cosmetics are subject to Regulation (EU) No. 1223/2009, which uniquely only allows the use of validated non-animal methods for safety confirmation. This means that of the 3Rs (Replacement, Reduction, Refinement), only Replacement alternatives are permitted. In contrast, industrial chemicals are regulated under REACH Regulation No. 1907/2006 (Registration, Evaluation, and restriction of Chemicals), which still permits animal testing, and in some cases, requires it. Although cosmetic ingredients are chemical substances, they are exempt from REACH concerning human health but not for environmental safety. With the EU’s Green Deal policy, environmental concerns, including those related to cosmetics, are gaining prominence. However, fewer alternatives have been developed and used in this area.

The training course provides insights into the regulatory framework and highlights differences/similarities in risk assessment of cosmetic ingredients for environmental and human health. It focuses on acute and chronic fish alternatives, existing gaps, and specific concerns related to UV filters, microplastics, polymers, endocrine activity, biodegradable formulation, new hazard classes, and urban wastewater. Through case studies and expert-led discussions, the course aims to bridge the existing gap between environmental and human health safety assessments.

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Application of the U-SENS assay for assessing skin sensitization potential of agrochemicals

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Skin sensitization testing is mandatory by global regulatory authorities as part of safety assessments for agrochemicals. There is substantial scientific evidence that has facilitated the proposal of an Adverse Outcome Pathway (AOP) for dermal sensitization, comprising four Key Events (KEs): (1) the binding of haptens to skin proteins, (2) the hapten-protein adduct-induced activation of keratinocytes, (3) the hapten-protein adduct-induced activation and maturation of dendritic cells and (4) the antigen presentation to naive specific T-cells. Different standardized testing protocols (*in chemico* and *in vitro*) have been developed, based on this AOP. It is generally accepted to combine methods covering at least two different KEs, within an IATA (integrated approaches to testing and assessment) or DA (defined approach), to obtain a reliable prediction replacing animal testing. However, both IATA and DA have been primarily evaluated using individual chemical components rather than mixtures or formulations.

Particularly, two cell-based methods have been developed to assess KE3 (OECD TG 442E): the U-SENS (CD86 surface marker quantification in U937 cell line) and h-CLAT (CD86 and CD54 surface markers quantification in THP-1 cell line) assays, with the latter being predominantly used for agrochemicals. To address this gap, we assessed the predictive ability of the U-SENS assay in evaluating the skin sensitization potential of agrochemicals, encompassing both the active ingredients (AIs) and their formulated products. We tested 12 formulations and 9 AIs, supplied by ATANOR S.C.A along with their associated *vivo* data. The performance metrics (accuracy, sensitivity, specificity, overestimation rate and underestimation rate) for hazard classification were calculated for the U-SENS assay relative to the *in vivo* data.

Additionally, we performed an *in silico* analysis of protein-binding alerts (PBAs), considering (a)biotic transformation, in the AIs and correlated these findings with the U-SENS assay results. CAS numbers and SMILES were imported into the OECD Toolbox v4.5 software in a batch run and the following profiling methods were then selected: “PBAs for skin sensitization by OASIS,” “PBAs for skin sensitization by OASIS, with Autoxidation simulator,” and “PBAs for skin sensitization by OASIS, with Skin Metabolism simulator”.

Predicting the PBAs in the AIs and their metabolites emphasized the importance of analyzing them before the U-SENS assay to reduce false negatives. The U-SENS assay exhibited a satisfactory performance in evaluating the skin sensitization potential of both formulations (accuracy: 75%) and AIs (accuracy: 67%). Moreover, the performance metrics obtained were comparable to or exceeded those reported for other substance types evaluated by the U-SENS assay, as well as for agro-



chemicals assessed with the h-CLAT method. This suggests that the U-SENS assay could be a promising application within a DA or IATA, as it allows the measurement of a single biomarker, thereby reducing time and resource requirements.

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Advancements in predictive modeling of skin sensitization: Enhancing the PredSkin platform with machine learning models for AOP key events and human data

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Skin sensitization is a critical endpoint in toxicological evaluation, as it assesses the potential of chemicals to induce allergic contact dermatitis, a major occupational disease. With frequent human exposure to chemicals in everyday life and increasing regulatory restrictions on animal testing, there is a growing demand for tools to predict skin sensitization potential. The PredSkin platform [1] is a web-based tool that utilizes Quantitative Structure-Activity Relationship (QSAR) models to classify sensitizing or non-sensitizing compounds.

As machine learning technology advances and new experimental datasets become available, there is a pressing need to update strategies to enhance predictive performance and applicability. For model updates, compounds from *in silico*, *in vivo*, and *in chemico* assays were selected to cover all key events of the Adverse Outcome Pathway (AOP) for skin sensitization. Datasets were sourced from reputable sources including NTP, NICEATM-ICVAM (<https://ice.ntp.niehs.nih.gov>), SkinSensDB [2], COSEU [3], and recent literature [4,5]. Five assays were incorporated: DPRA, h-CLAT/U-SENS, KeratinoSens/LuSens, LLNA, and Hu-

man data. After curation, the DPRA dataset comprised 560 compounds; h-CLAT/U-SENS, 2094 compounds; KeratinoSens/LuSens, 6467 compounds; LLNA, 1921 compounds; and the Human-HPPT dataset, 248 compounds. A time-split validation of new entries across the five datasets using PredSkin 3.0 models highlighted the need for model updates. A further data analysis of human data demonstrated that sixteen compounds changed classification, reinforcing previous findings.

These new datasets served as the foundation for predictive modeling using machine learning algorithms, including Random Forest, Support Vector Machine, Gradient Boosting, LightGBM, XGBoost, and deep learning approaches. Descriptor-based methods (MACCS, ECFP, RDKit) were integrated with descriptor-free approaches such as ChemBERTa [6] to optimize performance. In total, 21 models were developed for each dataset, combining these algorithms and descriptors. All models demonstrated high predictive accuracy, as assessed by balanced accuracy (BACC) and Matthews correlation coefficient (MCC). The highest-performing models for each dataset were: DPRA (ChemBERTa + Feedforward Neural Network, BACC = 0.70, MCC = 0.39), h-CLAT/U-SENS (ChemBERTa + Feedforward Neural Network, BACC = 0.70, MCC = 0.49), KeratinoSens/LuSens (MACCS + Support Vector Machine, BACC = 0.75, MCC = 0.40), LLNA (MACCS + Gradient Boosting, BACC = 0.69, MCC = 0.77), and Human-HPPT (ChemBERTa + Feedforward Neural Network, BACC = 0.67, MCC = 0.54).

An innovative model teacher distillation approach, where each skin sensitization model trained on distinct AOP endpoints served as a student model with the human dataset used for training, further boosted the predictive performance of the human model (BACC: 0.75, MCC: 0.51). Future work will focus on predicting sensitization potency and developing integrated strategies to enhance PredSkin's capabilities.

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Type II collagen extraction for scaffold development with 3D printing

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Introduction: Tissue engineering is an innovative field that combines engineering principles with health sciences to develop biological substitutes for damaged organs and tissues. A key aspect of this discipline is the production of scaffolds – three-dimensional structures that serve as biocompatible supports for cell growth and tissue regeneration. One of the main techniques for obtaining these scaffolds is 3D printing, with collagen being a primary material due to its abundance in the human body and its role in the extracellular matrix, where it provides mechanical support and facilitates metabolic exchanges.

Objective: This study aims to develop an effective methodology for extracting type II collagen from human costal cartilage. Methodology: Cartilage samples (CEP No. 1820, August 21, 2008) were subjected to various protocols and treatments to determine the most effective extraction method.

Results and discussion: Protocol 1: Crushed cartilage was immersed solely in acetic acid, yielding a collagen concentration of 5.5 mg/mL. When resuspended in phosphate-buffered saline (PBS) and incubated at different temperatures (4°C, 23°C, and 37°C), it exhibited low viscosity. Protocol 2: The samples underwent a pretreatment with PBS, sodium hydroxide, EDTA (ethylenediaminetetraacetic acid), and trypsin before extraction with acetic acid, as in Protocol 1. This resulted in a collagen concentration of 4.1 mg/mL, also with low viscosity upon resuspension. Protocol 3: A similar pretreatment to Protocol 2 was applied, but the enzymatic extraction step with trypsin was omitted. This method yielded a significantly higher collagen concentration of 18.1 mg/mL, though viscosity remained low upon resuspension. Protocol 4: This followed the same procedure as Protocol 3, but EDTA was applied only after resuspension to remove salts. The resulting collagen concentration was 2.1 mg/mL, with low viscosity after resuspension.

Conclusion: Protocol 3 demonstrated the best performance in terms of collagen yield. To achieve the necessary viscosity for use as a bioink in 3D printing of scaffolds, alginate will be incorporated into this protocol.

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Advancing the 3Rs: Evaluation of animal protein-free media for long-term cryopreservation of human PBMCs

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The use of fetal bovine serum (FBS) in cell culture and cryopreservation raises ethical concerns and potential risks. This study evaluates animal-protein-free media for long-term cryopreservation of human peripheral blood mononuclear cells (PBMCs), addressing the “Replacement” principle of the 3Rs (Replacement, Reduction, Refinement) in biomedical research. We assess the viability, yield, phenotype, and functionality of PBMCs cryopreserved in several commercially available animal-protein-free media, some with reduced or no dimethyl sulfoxide (DMSO), compared with an FBS-supplemented reference medium over 2 years.

PBMCs from 11 healthy volunteers were cryopreserved in a reference medium (FBS + 10% DMSO) and nine alternative serum-free media with varying DMSO concentrations. Cell viability and functionality were assessed at 3 weeks, 3 months, 6 months, 1 year, and 2 years post-freezing. Comprehensive readouts included cell viability and recovery, memory B cell FluoroSpot assay, and T-cell functionality assessed by three methods: cytokine secretion profiles in stimulated supernatants at all timepoints, IFN- γ /IL-4 FluoroSpot assay at 6 months, and intracellular cytokine staining at 30 months. Immunophenotyping was performed at 24 months. Multivariate analyses, including Principal Component Analysis and PARAFAC, were conducted to compare media performance.

PBMCs cryopreserved in CryoStor CS10 and NutriFreez D10 maintained high viability and functionality, comparable to the FBS10 reference medium, across all time points. Media with < 7.5% DMSO showed significant viability loss and were eliminated after initial assessments. Serum-free media with 10% DMSO preserved PBMC immune responses effectively. All three T-cell functionality assays showed consistent results, with cytokine profiles demonstrating stability over the 2-year period for CS10 and NutriFreez D10. Multivariate analyses revealed high similarity between CS10, NutriFreez D10, and FBS10 in preserving cellular integrity and functionality over time.

This comprehensive, long-term study demonstrates that CryoStor CS10 and NutriFreez D10, both serum-free media containing 10% DMSO, are viable alternatives to FBS-based media for long-term PBMC cryopreservation. These media ensure comparable cell viability and functionality, supporting their application in clinical and research settings. By eliminating animal-derived components, these alternatives address ethical concerns and reduce the risk of xenogeneic contamination, potentially improving the reliability and reproducibility of immunological studies while promoting animal welfare. This study represents a significant step towards establishing xeno-free media as a new standard in PBMC cryopreservation, aligning with the “Replacement” principle of the 3Rs and contributing to the reduction of animal use in biomedical research.

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Sexual hormone levels and computational toxicology as potential tools to assess the risk of occupational triazole exposure

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In a global scenario, the widespread and growing use of pesticides leads to the exposure of thousands of agricultural workers. The scarcity of studies contributes to the lack of data to implement regulations on these hazardous exposure conditions. In this context, risk assessment is crucial as an important and necessary process that aims to provide reliable scientific data, ensuring the prevention of toxic effects on human health, and both biomonitoring and computational toxicology (new approach methodology (NAM)) as an alternative to animal testing, contribute to the development of risk management strategies. Therefore, this study aimed to connect biomonitoring data to those produced by the computational tool to assess the risk of occupational exposure to triazole in coffee, the main Brazilian commodity. Participants of the study, men from the southern region of Minas Gerais, Brazil, were divided into two groups: farmworkers occupationally and environmentally exposed to pesticides from rural areas (n = 88) and urban residents (not occupationally exposed to pesticides), as a comparison group (n = 25). Three fungicides, cyproconazole, epoxiconazole, and triadimenol, were detected in the urine samples of the rural group. The urinary triazoles were detected in 35% of the workers showing absorption of these fungicides class. Androstenedione and testosterone hormones were significantly reduced in the farmworker group (Mann-Whitney test, $p < 0.0001$), even within the reference levels. Examining the potential impact of age as a confounding variable in sexual hormone levels, multiple approaches were applied, and the results demonstrated no significant age influence on testosterone levels. The curated high-throughput screening (HTS) data, via computational toxicology workflows from the Integrated Chemical Environment (ICE), confirm bioactivities related to steroid hormone metabolism at concentrations that overlap with those measured in humans. This study is extremely relevant, contributing significantly to reducing the enormous gaps in worker safety and health area, and subsidizing the implementation of more effective public policies for the protection of the health of these workers.

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Reconstructed human skin model as a tool to assess potential drug candidates for cutaneous leishmaniasis

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Leishmaniasis is considered by the World Health Organization (WHO) a Neglected Tropical Disease (NTD) and there are three main forms of the disease: Visceral leishmaniasis, Mucocutaneous leishmaniasis and the Cutaneous leishmaniasis (CL). The CL is the most common clinical form, characterized by skin lesions, mainly ulcers, erythema and/or nodular lymphangitis, that may leave permanent scars. Topical drug delivery may represent a great approach to treat CL lesions by offering significant advantages as the administration, fewer adverse effects and cost-effectiveness. However, topical formulations have to face the barriers of the skin, which is important to consider in CL due to the skin condition since the disease may cause open lesions or epithelial thickening. Thus, this study aimed to develop an in-house skin model as a tool to evaluate potential drug candidates for cutaneous leishmaniasis. Skin models containing different concentrations of *Leishmania amazonensis* infection were developed. Human primary fibroblasts were primarily infected with *L. amazonensis* and then the skin models were obtained by combining healthy and infected fibroblasts as well as human primary keratinocytes. The models were characterized by histological analysis with hematoxylin-eosin staining. It was observed that higher concentrations of infection caused a destruction of the skin models. The best ratio observed was 3:1 (healthy cells:infected cells), which caused some injuries in the epidermis but maintained the model preserved, and we also could observe the parasite in the dermis. The development of a physiologically relevant model that mimics the conditions of leishmania-infected skin is important for the elucidation of the early inflammatory mechanisms of CL and offers a tool for evaluating drug candidates for the topical treatment of this disease.

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Advancing transformer-based AI for chemical risk assessment

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New Approach Methodologies (NAMs) seek to reduce reliance on animal testing by promoting alternative, efficient strategies for assessing chemical safety. Traditional Quantitative Structure-Activity Relationship (QSAR) models, while valuable, often predict properties in isolation, limiting their ability to fully leverage complex data across multiple chemical properties. In this study, we explore a transformer-based model designed to predict sequences of property values simultaneously, allowing for a more comprehensive approach that could support NAM principles in public health. This model also enables exploration of counterfactual scenarios, such as, “If a chemical has property X, what is the likelihood it has property Y?” – a capability that may inform efficient testing strategies and deepen our understanding of chemical-property relationships.

Our encoder-decoder transformer model tokenizes Self-Referencing Embedded Strings (SELFIES) of molecules and sequences of property-value pairs. Trained on the extensive ChemHarmony dataset, with information on over 118 million substances, the model leverages embeddings of property-tokens, known properties, and molecular structures to make robust predictions. To evaluate its performance, we benchmarked the model against established datasets, including BBBP, HIV, and SIDER. Preliminary results show that our model performs favorably in comparison to published QSAR models on these benchmarks, suggesting strong potential for conditional predictions and transfer learning across properties.

This “property-value-transformer” model offers a promising step forward in QSAR methodologies and NAM applications, with implications for more efficient and ethical chemical testing strategies.

Key Contributions: Introduction of a multi-task framework for simultaneous prediction of molecular properties supporting NAM.

Strong preliminary results on benchmark datasets (BBBP, HIV, SIDER) demonstrating improved performance on conditional predictions.

Exploration of conditional inference capabilities, with potential applications in integrated chemical testing for public health.

Integrate BioBricks.ai for Multi-Modal Learning: Utilize curated datasets to incorporate diverse data modalities for refined toxicity assessments. BioBricks.ai is a dvc data management tool we developed.

Aim to Deploy an Interactive AI Copilot: Develop a web-based API for real-time chemical property predictions, uncertainty estimates, and interactive exploration.

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Spirulina maxima: An alternative to the use of fetal bovine serum in cell culture

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Fetal Bovine Serum (FBS) is widely used in cell culture due to its large composition in growth factors and essential nutrients for cell development and proliferation. It is produced through venipuncture, or cardiac puncture, in a still alive calf, in which the blood goes through several processes in order to obtain the serum. Therefore, the production of this serum is surrounded by ethical, economic and environmental concerns, as well as limitations such as the nutritional variability between batches and the risk of contamination. However, microalgae, more specifically the helicoidal and filamentous cyanobacteria *Spirulina maxima* from *Oscillatoriaceae* family, appears as an innovative and promising alternative for FBS, due to their potential to provide proteins, fatty acids, vitamins and other bioactive compounds. Hence, we here hypothesize that this cyanobacterium could be a substitute for the use of Fetal Bovine Serum in Mammalian Cells Culture. To evaluate this hypothesis, a human chondrocyte cell line (SW1353) was cultivated using F15, with 10% FBS in 37°C and 5% CO₂. *Spirulina maxima* extract, in a powder form (2 µg/mL) was resuspended in F15 cell culture medium with 0,1% DMSO and lysed by ultrasound and sterile filtered before use. Chondrocytes were cultured in F15 cell culture medium supplemented with 1% and 10% of FBS and different concentrations of *Spirulina maxima* (2,5; 5 and 10 µg/mL). Cellular viability was measured using a resazurin-based assay from 24 hours up to 240 hours. The results of the viability experiment suggest that condition C (F15 + 10 µg/mL + 1% SFB) behaves similarly to condition E (F15 + 10% SFB) over time, indicating that the addition of the algae extract can provide cell support comparable to that of fetal bovine serum. Therefore, the best performance, or more similar to the use of 10% FBS, was with 10 µg/mL in a cell culture medium supplemented with 1% of FBS. This similarity reinforces the potential of algae as a promising alternative for partial or total replacement of SFB, reducing dependence on animal products and encouraging the development of more sustainable culture media. The main conclusion is that *Spirulina maxima* in 10 µg/mL in a cell culture medium supplemented with 1% of FBS demonstrates a similar cell maintenance statistically and morphologically to the same cell culture medium with 10% of FBS.

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Exploring alternative methods in probiotic research for colitis: Beyond traditional animal models

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Recent research on probiotics and their impact on colitis models has centered on three key areas: probiotic efficacy in colitis, alternative probiotic delivery methods, and microbial composition modulation. Various probiotic strains and formulations have demonstrated potential in alleviating colitis symptoms by modulating gut microbiota and immune responses. Understanding these mechanisms is essential for developing effective therapeutic strategies for inflammatory bowel disease (IBD). However, the traditional approach to evaluating probiotic efficacy has relied heavily on animal models, which involve inducing colitis through various methods that mimic human IBD conditions. Among the most widely used chemical induction methods, trinitrobenzenesulfonic acid is administered intrarectally, often in combination with ethanol, to trigger an inflammatory response resembling Crohn's disease. Similarly, acetic acid (AA) is used to cause colonic irritation through rectal instillation. Another common model, dextran sulfate sodium (DSS), is administered orally to induce mucosal damage, closely simulating ulcerative colitis. Additionally, bacterial models involve colonizing germ-free animals with specific enteric bacteria to investigate the role of gut microbiota in colitis development. While these methods have significantly contributed to IBD research, they are associated with considerable animal distress, suffering, and ethical concerns, reinforcing the urgent need for alternative approaches. The 3Rs principle – Replacement, Reduction, and Refinement – guides researchers toward minimizing animal use while maintaining scientific rigor. In response, alternative models have emerged to complement or replace traditional animal studies, providing innovative ways to evaluate probiotic effects. *In vitro* models, such as those using Caco-2 intestinal epithelial cells, enable researchers to study probiotic interactions with the gut barrier without relying on live animals. These models offer valuable insights into cell integrity, immune signaling, and microbial adhesion, facilitating the identification of probiotic strains with protective properties. *Ex vivo* approaches, which involve using fermentation supernatants from probiotic cultures, allow researchers to examine probiotic-derived metabolites and their potential role in modulating inflammation, bridging the gap between cell-based studies and whole-organism effects. Non-mammalian models provide another promising alternative. Probiotic yeasts have demonstrated anti-inflammatory properties and microbiota-modulating effects, offering a viable reduction of mammalian models. Yeast-based systems allow researchers to explore host-microbe interactions in a simplified, ethically favorable context. While the DSS-induced colitis mouse model remains a widely used standard, alternative models provide valuable complementary approaches, aligning with both scientific and ethical imperatives. Reducing dependence on animal testing not only adheres to evolving regulatory guidelines but also enhances methodological diversity, improving the reliability and applicability of

probiotic research. The adoption of these alternatives not only aligns with ethical principles aimed at reducing animal use in scientific research but also expands methodological diversity, contributing to the broader goal of improving treatment strategies for inflammatory bowel disease.

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Communities for open research synthesis (CORS): Advancing systematic review culture in preclinical research

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The Communities for Open Research Synthesis (CORS) project develops a targeted framework to initiate systemic change in how preclinical research is conducted, synthesized, and translated into improved human health outcomes. Systematic reviews and meta-analyses play a crucial role in enhancing research quality by evaluating evidence reliability, identifying knowledge gaps, and informing future studies. These approaches promote the 3Rs by refining study designs, reducing unnecessary duplication, and validating alternative methods to animal research.

CORS employs a three-pillar strategy to integrate systematic reviews into preclinical research: (1) Education – delivering open-access training, e-learning modules, and a train-the-trainer program to build capacity; (2) Infrastructure – developing and optimizing systematic review tools, including enhancements to the Systematic Review Facility (SyRF) software; and (3) Community – establishing networks for interdisciplinary collaboration and fostering a culture of evidence synthesis.

To inform CORS implementation, we conducted a survey at Charité – Universitätsmedizin Berlin, one of Europe's largest university medical centers, assessing awareness, attitudes, and practices regarding systematic reviews among researchers, clinicians, and support staff. Follow-up interviews provided in-depth insights into key barriers (e.g., lack of training, time constraints, and limited institutional support) and enablers (e.g., growing interest in reproducibility, methodological training, and access to systematic review tools). Findings from this study guide strategies to support researchers in conducting systematic reviews and incorporating synthesizable practices into preclinical research.

Building on this, we are extending our survey to university medical centers across Germany to refine a nationwide implementation strategy with our pilot program. By embedding systematic reviews into the research pipeline, CORS advances high-quality, transparent, and reproducible research while aligning with the One Health framework – bridging human, animal, and environmental health sciences to drive impactful biomedical research.

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How to increase the acceptance of machine learning-based QSAR-like *in silico* NAMs for nanomaterials?

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New Generation Risk Assessment (NGRA) requires integrated approaches like Integrated Approaches to Testing and Assessment (IATAs) and Adverse Outcome Pathways (AOPs). Efficient testing strategies should also incorporate *in silico* New Approach Methods (*in silico* NAMs) to reduce cost and time. Over 200 *in silico* NAMs, including nano-QSARs, have been proposed for nanomaterial risk assessment. Nano-QSARs predict toxicity based on molecular and supramolecular “nano-structure,” interactions, and microenvironment influence.

However, building trust in computational models remains a challenge. While the OECD QSAR Assessment Framework provides validation guidelines, acceptance of models may require additional criteria. We propose four criteria to improve acceptance, particularly in the regulatory context:

1. Reliability (per OECD QSAR Framework): well-defined endpoints, clear algorithms, applicability domains, validation, and mechanistic interpretation.
2. Toxicological Relevance: how well the model contributes to understanding toxicity mechanisms.
3. Regulatory Relevance: potential use in regulatory frameworks.
4. User-Friendliness: accessibility for toxicologists without computational expertise.

The importance of these criteria is illustrated by three case studies, based on *in silico* NAMs, including:

- a) a novel classification model for predicting water solubility of metal and metal oxide nanoparticles;
- b) a set of models for predicting genotoxicity (comet and micronucleus tests) for metal oxide nanoparticles;
- c) a set of models for predicting lung toxicity of multiwalled carbon nanotubes (MWCNTs) at the molecular level (utilizing transcriptomics data).

For toxicological relevance, solubility prediction was deemed relevant but could be improved by assessing solubility in biological fluids. Genotoxicity models would be more relevant if they incorporated cell penetration. The MWCNT toxicity models were highly relevant, aligning with the OECD-approved AOP-33.

In terms of regulatory relevance, model (a) supported nanomaterial grouping per IATA guidelines. Model (b) aligned with EFSA genotoxicity guidance. Model (c) had lower regulatory relevance but was linked to the first OECD-approved AOP for nanomaterials, which could facilitate MWCNT classification based on aspect ratio.

User-friendliness was the weakest aspect for model (a), available only as Python code. Model (b) was implemented in the user-friendly but commercial nQTB software. Model (c) had the highest usability, available as an easy-to-use web tool.

Validation ensures model quality but does not guarantee acceptance. The OECD QSAR Framework provides validation, but additional criteria help build trust. Although all models scored highly in reliability, assessments of toxicological relevance, regulatory relevance, and user-friendliness varied. These findings may guide *in silico* NAMs developers in enhancing their models acceptance among researchers and regulators.

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Biomarkers of teratogenicity using stromal cells for the development of new approach methodologies (NAMs)

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Exposure to chemicals during pregnancy may be linked to adverse effects on embryonic development. The OECD 414 (2018) test is the gold standard for assessing developmental and reproductive toxicity (DART), but it requires nearly 1,000 animals per study, making it costly, time-consuming, and potentially unreliable for predicting human responses. Consequently, there is a demand for New Approach Methodologies (NAMs) to reduce animal testing and enhance the accuracy of DART assessments. Human dental stromal cells represent a promising alternative source of adult stem cells, presenting high plasticity and multipotency while being readily available from discarded biological materials, such as extracted teeth. In this context, the present study aimed to develop a teratogen screening platform using dental stromal cells as a human-based model. Stromal cells from apical papilla (SCAPs) were isolated, cultured, and characterized by flow cytometry. The cells were exposed to classical teratogenic compounds 5-fluorouracil (0.1-360 µg/mL), methotrexate and cyclophosphamide (0.1-1000 µg/mL), and non-teratogenic compound folic acid (0.16-250 µg/mL) to determine the CV80 value using the MTT assay. Additionally, expression of the biomarker Nestin, OCT-3/4, and Stro-1 were evaluated by flow cytometry at 48 hours, while CD90 and CD105 were analyzed over time intervals from 24 to 120 hours. Non-exposed SCAPs exhibited a positive phenotype to CD90, CD105, Stro-1, Nanog, OCT-3/4, and Nestin, while CD34 expression was negative. No cytotoxicity was observed at any concentration after 24 hours of exposure to 5-FU, MTX, CTX, and folic acid. However, the CV80 value was determined to be 13.47 µg/mL (Tukey, ANOVA, $p < 0.05$) after 48 hours of exposure to 5-FU. The CV80-48h values for folic acid, MTX, and CTX could not be established. No modulation was observed in the expression of Nestin, OCT-3/4, Stro-1, and CD90 following exposure to 5-FU. However, a concentration and time-dependent reduction in CD105 expression was demonstrated when SCAPs were exposed to 5-FU, MTX, and CTX after 72 and 120 hours. In contrast, the non-teratogenic compound folic acid did not alter the expression of this mesenchymal biomarker. This study highlights the potential of human dental stromal cells as a promising tool for developing NAMs, particularly due to the specific association between teratogenic compounds and CD105 regulation. The reduction in CD105 expression decreases its interaction with the ALK-1 receptor, which may lead to the activation of the SMAD 1/5/8 pathway, ultimately inhibiting cell proliferation, migration, and angiogenesis. This downregulation can also compromise the TGF-β signaling pathway and, consequently, affect angiogenesis, resembling the teratogenic mechanism of thalidomide. This finding highlights CD105 as a key teratogenicity biomarker, bridging data gaps. Given its role in MSC biology, chemically induced alterations in SCAPs may signal teratogenic potential, making CD105 a promising target for screening teratogens.

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Assessment of mycotoxin contamination in rice and rice bran from Kirinyaga, Kisumu, and Kwale counties, Kenya

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Rice (*Oryza sativa*) is a global staple food, significant for the economies and diets of many regions, including Kenya, where it is a primary food source in the coastal and Northern regions. Rice provides carbohydrates, vitamins, and minerals and is an economic resource, with surplus sold to finance various household activities. In Kenyan rice growing regions of Kirinyaga, Kisumu, Kwale and others, small-scale farmers produce approximately 150,000 MT annually. However, rice and its byproduct, rice bran, can be contaminated by mycotoxins, posing significant public health and economic risks. Previous studies have detected mycotoxins in some Kenyan rice samples, but comprehensive data on fungal and mycotoxin contamination in Kenyan rice and rice bran are scarce. We aimed to identify mycotoxin-producing microorganisms and determine mycotoxin levels in rice and rice bran from Kirinyaga, Kisumu, and Kwale counties. The findings are essential for advising farmers on appropriate harvesting handling to reduce wastage and improve rice quality. The study employed random and composite sampling techniques to collect rice paddy samples, which were then polished to separate the bran from the rice, followed by laboratory analysis of mycotoxins. Eighteen rice paddy samples (six from each county) were cultured using Sabouraud's Dextrose Agar at 25°C and 37°C to determine fungal growth. Fungi were examined for characteristic coloration and growth patterns, and portions were stained and microscopically examined for identification. Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) analyzed Aflatoxins (B1, B2, G1, and G2) and Fumonisin (B1 and B2). The results revealed varying, but insignificant levels of mycotoxin across regions that were inconclusive in terms of contamination. All rice grain samples were negative for Aflatoxins, however, a few were positive for Fumonisin B2 when compared to the East African Standards limits. Fumonisin was detected in one sample each in Kwale (174.5 ± 2.75 ppb) and Kirinyaga (166.8 ± 1.00 ppb). Rice bran analysis revealed Aflatoxin B1 contamination in two samples from Kwale (5.0 and 10.17 ppb, respectively). The cultivated samples to isolate and identify fungal species did not show any fungal growth. These findings indicate insignificant mycotoxin contamination in the study regions, likely due to improved postharvest handling and milling processes. This is significant because it provides critical data on the safety and hygiene of rice and rice bran food components essential for human health. This finding could lead to enhanced harvesting and storage practices, improved health outcomes, and economic benefits for Kenyan rice farmers. Further research is needed to investigate the long-term effects of rice bran consumption on infant nutrition and to develop effective strategies for integrating it into local diets.

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Standardization of a 3D bioprinted skin model for safety and efficacy evaluation tests

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In vitro cell cultures of human tissues are used as an alternative method to animal testing for the initial assessment of the safety and efficacy of a new active ingredient. These tests are usually performed on monolayer 2D models of human skin cells. However, evidence suggests that 2D *in vitro* cell cultures have several disadvantages, including changes in cell shape, cell function, cell responses and a lack of interactions between cells. Unlike monolayer tests, which are limited to water-soluble substances, 3D models, such as reconstructed human skin (RHS), can be used to test any type of substance, including finished products. Reconstructed 3D skin models are considered physiologically relevant and bioprinters yield better results regarding tissue organization and variability between models. Therefore, this study proposed a standardization of an in-house 3D full thickness skin model using the Genesis bioprinter (3DBS, Brazil). The dermis was first prepared, containing collagen and human primary fibroblasts. The bioprinting was performed with a helical movement. After 24 hours, the epidermis was bioprinted, using the bioink containing human primary keratinocytes. The models were cultured in an air-liquid interface for 7 days to ensure the stratification and differentiation of the epidermis. The models were characterized by histological analysis with hematoxylin-eosin staining. The bioprinted skin demonstrated enhanced structural complexity and cell differentiation when compared to the manual RHS previously obtained by our group, highlighting its potential for more reliable safety and efficacy assessments of active ingredients. Other assays should be performed to confirm these results and provide a more accurate analysis. By overcoming the limitations of monolayer tests, this approach offers a more physiologically relevant model for *in vitro* studies, leading to improved methodologies in dermatological and pharmaceutical research.

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Preliminary evaluation of requirements and acceptance of *in silico* models in Brazilian regulation

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Advancements in New Alternative Methodologies (NAMs) have driven the development of *in silico* models, such as Quantitative Structure-Activity Relationship (QSAR) models, reducing time, costs, and animal use. However, regulatory integration remains challenging, especially in developing countries. Although Brazil follows international guidelines, specific regulations for these models are lacking. Despite not being a regulatory body, the Organisation for Economic Co-operation and Development (OECD) has played a key role in promoting *in silico* models. Series No. 69 (2007/2014) established (Q)SAR validation principles, while Series No. 386 and No. 405 (2024) updated the (Q)SAR Assessment Framework (QAF), enhancing reproducibility and acceptance. This research surveyed Brazilian regulatory guidelines on *in silico* models, focusing on the National Health Surveillance Agency (ANVISA), the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), and the National Ethics Committee for Animal Use (CONCEA), comparing them with international standards. A search was conducted in agency repositories between January and March 2025 using the keywords “*in silico*” and “(Q)SAR.” Results indicate gaps in the regulatory acceptance of *in silico* methodologies in Brazil. While CONCEA promotes alternatives to animal testing, it lacks specific guidelines for these models. Similarly, IBAMA does not regulate *in silico* models for environmental risk assessment. ANVISA, responsible for regulating pharmaceuticals, food, cosmetics, health products, sanitizers, tobacco, and pesticides, has gradually incorporated *in silico* approaches. RDC 294/2019 updated toxicological evaluations for pesticides and wood preservatives, requiring applicants to justify acceptance criteria using scientific data and to assess (Q)SAR models on a case-by-case basis. RDC 53/2015 recognizes (Q)SAR for predicting chemical degradation impurities, following ICH M7 guidelines, which require two complementary prediction methods to assess mutagenicity, in line with OECD. The absence of structural alerts allows impurity classification as non-mutagenic without additional testing. Globally, many regulatory agencies have adopted *in silico* methodologies. The REACH regulation (Registration, Evaluation, Authorization, and Restriction of Chemicals) from ECHA has been included *in silico* models since 2007 to assess toxicity and environmental impact, aiming to reduce animal testing. In collaboration with the OECD, the QSAR Toolbox was developed to predict toxicological properties. It promotes the use of *in silico* models following OECD guidelines outlined in the Guidance on Information Requirements and Chemical Safety Assessment. Furthermore, the FDA has promoted computational toxicology tools. In its 2022 report, Successes and Opportunities in Modeling & Simulation, M&S applications were highlighted. In 2023, the FDA issued guidance assessing the credibility of computational modeling in medical device submissions. To advance *in silico* methodologies, Brazil must modernize its regulatory framework. This includes adapting OECD guidelines to the national context, supporting sector harmonization, and investing in technical and regulatory training. These steps will provide credibility to the use of *in silico* methods and align Brazil with international best practices, enhancing global competitiveness.

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Human corneal stromal stem cells from non-transplantable corneas: A step toward animal-free eye research

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Ocular research has long relied on various animal models to assess both healthy and pathological conditions. Despite significant advancements in replacing animals in experimental eye research, many *in vitro* and *ex vivo* methods still depend on animal-derived cells or tissues (e.g., cell lines, bovine corneas). This underscores the growing need for more physiologically relevant human cell sources. Our group has standardized a protocol for obtaining high-yield human corneal stromal stem cells (hCSCs), which can be further differentiated into other ocular cell types. However, while we have successfully isolated these cells from corneas discarded after visceration surgeries, the availability and quality of such tissues are often limited, primarily due to the traumatic causes leading to eyeball removal. Meanwhile, numerous corneal tissues intended for transplantation are discarded weekly due to expiration or minor defects that compromise their suitability for clinical use. Although unsuitable for transplantation, these tissues could serve as a valuable source of hCSCs for experimental eye research, offering a physiologically relevant alternative to animal-derived tissues.

This study aims to compare the phenotypic characteristics of hCSCs obtained from fresh corneas through surgical procedures and those derived from expired corneas designated for transplantation, following ethical approval. Using an explant culture method without enzymatic digestion, cells were isolated, cultured, and expanded *in vitro* according to our standardized protocol. hCSCs from both sources were characterized by flow cytometry, assessing the expression of CD73, CD90, CD105, CD34, CD45, STRO-1, Nanog, OCT3/4, Nestin, Pax6, and GFAP at passages 2 (P2), 4 (P4), and 6 (P6). Cells from both sources exhibited high expression of mesenchymal stem cell markers (CD90, CD73, and CD105) across all tested passages, confirming their stem cell characteristics and differentiation potential. CD34 expression was detectable at P2 but declined sharply at P4 and P6, suggesting the initial presence of keratocytes that were gradually eliminated over successive passages, leading to a purer MSC-like population. CD45 and STRO-1 remained consistently negative. Additionally, hCSCs expressed Pax6 and Nestin at all passages, indicating ocular lineage commitment and neural progenitor traits, respectively. Differentiation assays further demonstrated that cells from both sources successfully differentiated into osteogenic and adipogenic lineages, confirming their multipotency.

Taken together, these findings demonstrate that corneas discarded from eye banks provide a sustainable, cost-effective, and high-yield

source of hCSCs for experimental eye research, with no difference regarding the fresh surgery tissue, facilitating the use of human-derived cells as an alternative to animal-derived tissues.

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Substitution of fetal bovine serum for platelet-rich plasma in the isolation of extracellular vesicles from umbilical cord mesenchymal stromal cells

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The use of fetal bovine serum (FBS) in cell cultures is controversial due to the way it is obtained, through cardiac puncture of bovine fetuses without anesthesia, and the high variation between batches. In addition, its animal origin may restrict its application in clinical contexts. Platelet-rich plasma (PRP), a platelet concentrate in a small volume of plasma, is a possible alternative to SFB, with promising results in terms of its ability to promote cell proliferation. Furthermore, there is evidence that extracellular vesicles (EVs) originating from mesenchymal stromal cells (MSCs) perform functions analogous to those of the MSCs themselves. They carry bioactive molecules destined for the target cells, capable of modifying their phenotype or functional behavior. Our goal is to modulate the secretion and content of EVs secreted by umbilical cord-derived MSCs through culture media supplemented with different concentrations of PRP, as a substitute for SFB, with the aim of increasing their therapeutic potential so that they can be used later in the development of a new treatment for osteoarthritis. EVs were isolated from the supernatant of umbilical cord cell cultures by ultracentrifugation and characterized for size and concentration using the Nanoparticle Tracking Analysis (NTA) technique. Additional analyses, including Transmission Electron Microscopy, to assess morphology; qPCR, for expression of miRNAs modified during osteoarthritis (miR-140 and miR-337); and Western Blot, for expression of typical vesicle proteins (CD9, CD63 and CD81), are underway. The NTA technique indicated that it was feasible to isolate similar quantities of extracellular vesicles from the culture supernatant of umbilical cord MSC when supplemented with 10% fetal bovine serum (FBS) compared to supplementation with 2.5% PRP ($p > 0.05$). In addition, it was found that the isolated vesicles were similar in size in both scenarios (SFB: 131 ± 11 nm; PRP: 126 ± 21 nm). We can conclude that there is a possibility of replacing the use of SFB, a xenogeneic product, with PRP, of human origin, to obtain extracellular vesicles from human umbilical cord MSC.

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Development of low-cost intestine-on-a-chip for pharmacokinetic assays

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Background: Intestine-on-a-chip (IoC) models made of PDMS pose challenges in ADME assays due to the adsorption of low-molecular-weight hydrophobic compounds, compromising the accuracy of pharmacokinetic and dose-response studies.

Objective: To develop a low-cost microchip using an alternative material to PDMS, minimizing interference in pharmacokinetic assays. The device should support epithelial and endothelial co-culture and be validated through permeability tests to assess barrier integrity and absorption capacity.

Methods: The device was fabricated using laser-cut thermoplastic material. Caco-2 cells were cultured in the upper channel, while HUVEC cells were cultured in the lower channel for 5 and 10 days. Barrier integrity was evaluated using the phenol red assay, and absorption capacity was assessed using caffeine.

Results: The IoC consists of two curved microfluidic channels separated by a semipermeable porous membrane. The fabrication cost is 1.5 times lower than that of a PDMS-based chip. Computational simulations indicated that at a flow rate of 8 $\mu\text{L}/\text{min}$, the shear stress of 1.5×10^{-2} dyne/cm^2 aligns with physiological intestinal values. Permeability assays with phenol red demonstrated that, after five days, the Caco-2/HUVEC co-culture formed a cellular barrier with permeability of $8.45 \times 10^{-6} \text{cm}/\text{s}$, comparable to the Transwell model ($3.58 \times 10^{-6} \text{cm}/\text{s}$). Caffeine absorption between 15 and 90 min also showed consistency with Transwell results.

Conclusion: The developed IoC efficiently replicates intestinal absorption and drug evaluation in a dynamic system. Its ability to mimic intestinal physiology reinforces its potential as a more accurate tool for pharmacokinetic studies.

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Science engagement in African mother tongues as a method of increasing scientific literacy

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Effective science engagement facilitates integration of scientific knowledge into everyday choices and decisions, evidence-based decision making, and in the understanding required to prioritize and invest in biomedical research as part of essential government expenditure. The internet and the social media platforms within it have been useful tools for science engagement by providing ready and credible access to information about science, scientific discoveries, and information about human health to users across the globe. These resources have expanded access to education professionals who encompass a diverse range of skills, expertise, and linguistic knowledge thereby creating informal education resources that can supplement understanding and provide clarity about scientific and health-related knowledge. These informal education resources are of increased importance because according to UN reports, 40 per cent of the world's population does not have access to education in a language they speak or understand. Against this landscape, informal scientific education and science engagement in various languages emerge as an underexplored and underused frontier of education resource development across the globe. In this poster, I aim to discuss how using African mother-tongues and non-English languages for science engagement on social media platforms presents a powerful tool that can: 1) Increase scientific literacy across education barriers, 2) Facilitate language revitalization of various underused mother-tongues, 3) Integrate modern knowledge and traditional knowledges, and 4) enable fertile possibilities for interdisciplinary scholarship. Taken together, science engagement in mother tongues offers a multifaceted approach to increase scientific literacy especially in countries where much of the population have varied access to education resources.

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Trends in private funding of alternative methods: ARDF's 30 years of building community and supporting NAMs research

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The Alternatives Research & Development Foundation (ARDF) was established in 1993 to fund research to develop innovative methods that replace or reduce the use of animals in research, testing, and education. One of the longest-running efforts of its kind, ARDF's grant programs fund investigator-initiated research across a broad – and evolving – range of scientific areas with the aim of supporting projects that have both high scientific merit and potential to contribute to the replacement of animal models.

Award amounts have increased from a maximum of \$30,000 in the initial funding cycle (1994) to \$50,000 in 2025 for one-year grants. The program invites proposals from non-profit educational or research institutions worldwide and has made awards to institutions in 8 countries, though the majority have been to US-based institutions. The program has awarded about \$5.3 million in funding since its inception.

A review of grants awarded during the foundation's first 30 years (1994-2023) suggests that its research funding has contributed to scientific progress in several areas. Early investments supported education, the development of novel methods for *in vitro* production of monoclonal antibodies, and research on infectious diseases. Other areas, including toxicology, cancer research, and drug development, have been continuously supported throughout this period.

The grant portfolio also reflects the considerable technological advancements over this time with development of increasingly sophisticated *in vitro* and *in silico* models. The first grant for the development of a microphysiological system (MPS) was awarded in 2015. Since then, organ-on-chip models and organoids have become more prevalent, and proposals incorporating computational modeling and AI are increasingly common. The number of applications submitted in recent years has also risen dramatically, suggesting a rapid increase in interest in non-animal research methods.

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On the road to speeding up – Accelerating the transition to animal-free safety assessment by examining the European Commission roadmap

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As publics worldwide are increasingly re-examining the connection between human and non-human animals, the topic of animal testing has also gained attention for its problematic practices. Despite its questionable relevance for human health, its poor replication standards, and large variability, animal studies are still deeply entrenched in science production and safety assessment and are seen as a “gold standard”.

In the European Union (EU), *de jure* animal models are to be used only as a last resort, but *de facto* animal testing is much more widespread than what the legal guidelines mandate. Officially, the policy of the EU is to fully transition away from animal testing. Yet the slow pace of this transition has triggered a number of initiatives from the EU parliament and EU citizens. In the EU, animal testing has been the topic of multiple European citizen initiatives condemning it. The EU parliament also urged the EC to accelerate the pace of the transition to animal-free science.

In response to this, the EC has committed to developing a roadmap outlining how it aims to manage this transition. The EU is uniquely positioned to implement a transition to animal-free safety assessment and has many opportunities to align an ambitious goal of phasing out animal testing in the EU with other EU policies such as the Green Industrial Plan or its Chemicals for Sustainability strategy. Its close ties with the USA and educated workforce means it has great potential to implement an ambitious strategy. Since 2024, key stakeholder meetings, a call for evidence, and surveys have been conducted to inform the roadmap drafting process in order to publish the finished proposal in 2026.

A transdisciplinary and international consortium based mainly in the Netherlands, the SAFE consortium, has been active in studying the EC roadmap process. Through the use of its action research methodology, the consortium has been learning about the EC's strategy on developing its roadmap and has been making efforts to influence the process. This presentation will outline the consortium's strategy to influence the transition and the main barriers and opportunities to accelerating the transition that have been observed, and discuss the preliminary findings on what the most effective strategies are to engage with policymakers. It will shed light on practical steps to engage with stakeholders in Global South or Global North, and theoretical frameworks for guiding such engagement.

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MatTek's EpiDerm™ and EpiOcular™: Comprehensive validation and expanding applications in alternative methods

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MatTek produces an extensive portfolio of advanced reconstructed human tissue models – skin, eye, respiratory tract, oral mucosa, vaginal mucosa, and intestinal epithelium – widely used in safety and efficacy assessments. Among these innovative tissues, the EpiDerm™ (reconstructed human epidermis) and EpiOcular™ (reconstructed human cornea-like epithelium) models have notably transformed global regulatory toxicology by offering scientifically validated, ethical, and animal-free alternatives to traditional *in vivo* testing. Recognized internationally for their high predictive accuracy and relevance to human health, these models facilitate robust hazard evaluations across cosmetics, pharmaceuticals, chemicals, and medical devices, significantly enhancing both consumer safety and regulatory acceptance worldwide.

The EpiDerm™ model is fully validated and accepted for skin irritation (OECD TG 439), skin corrosion (OECD TG 431), and phototoxicity (OECD TG 498), effectively replacing traditional animal-based tests such as the Draize rabbit skin irritation/corrosion assays (OECD TG 404) and phototoxicity methods. In addition, EpiDerm™ is recognized under ISO 10993-23 as a validated, non-animal method for assessing irritation potential in medical devices, reinforcing its essential role in global regulatory evaluations across various industries.

Similarly, the EpiOcular™ model is internationally recognized under OECD TG 492 for ocular irritation testing, offering a reliable and ethical alternative to the traditional Draize rabbit eye irritation test (OECD TG 405). EpiOcular™ continues to be a key model in ocular safety testing, enabling accurate predictions that help optimize regulatory processes globally.

To further broaden the applicability of its models, MatTek is advancing additional validations, including the Epi2SensA method, designed to detect molecular biomarkers involved in early phases of skin sensitization. After undergoing rigorous optimization and pre-validation, Epi2SensA is in process of “me-too” validation for inclusion in the OECD TG 442D. This method provides a precise, *in vitro* means of identifying skin sensitizers, supporting efforts to reduce reliance on traditional animal testing.

Furthermore, MatTek has successfully developed the EpiOcular™ time-to-toxicity method, fully meeting the performance criteria set by the OECD expert group overseeing TG 492B. This innovative approach enables the accurate classification of both liquid and solid substances into the three UN GHS ocular hazard categories: No Category, Category 2, and Category 1. The adoption of this method marks a significant advancement in ocular safety assessments, enhancing global regulatory harmonization and improving the precision of eye irritation tests.

MatTek validated models, together with ongoing validations in the OECD pipeline, are widely accepted by leading regulatory agencies, including the EU Cosmetics Directive, REACH, US EPA, FDA, Health Canada, and ANVISA. These innovations have drastically reduced animal use, sparing thousands of animals annually from painful irritancy tests. By promoting high standards in ethical scientific innovation, MatTek continues to lead the way in advancing safety assessments while ensuring robust consumer protection and animal welfare.

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Synthesis and characterization of gelatin methacrylate-based hydrogels for brain cell culture in a microfluidic device

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In native tissue, cells exist in dynamic 3D environments, where their interactions with the microenvironment play a crucial role in tissue morphogenesis and function. While significant discoveries have been achieved through 2D culture, there are often substantial differences in cellular responses when comparing 2D to 3D culture, including variations in cell polarization. To explore the cell-matrix interactions that drive cell differentiation and self-assembly *in vitro*, it is essential to mimic the dynamic nature of the extracellular matrix. Hydrogels have emerged as valuable materials in this regard due to their properties that closely resemble the extracellular matrix. One promising biomaterial in tissue engineering is gelatin methacrylate (GelMA), which is produced by modifying gelatin through a reaction with methacrylic anhydride. GelMA is favored for its excellent biocompatibility, strong cell adhesion, adjustable physicochemical properties (depending on the degree of methacrylate substitution), and ability to undergo photo-crosslinking when exposed to UV light. Using GelMA combined with basement membrane extracts may enhance the mimicry of a microenvironment suitable for 3D culture. Also, the association of hydrogels in microfluidic devices has been increasing the accuracy of 3D cultures by incorporating shear stress and flow, which enables the creation of dynamic environments, the so-called microphysiological systems. In this context, we explored the use of GelMA 7.5% (w/v) alone or in a 50:50 ratio with basement membrane extract (Geltrex[®]), identified as GM7.5 and GM7.5_GX, respectively. The GelMA concentration for these tests (7.5%) was chosen based on preliminary tests of the success rate of its application underflow (1-5 μ L/min) in a hydrogel-based microfluidic device. Hydrogels were assessed for mechanical properties (compressive and elastic modulus), porosity (scanning electron microscopy), and cell viability of a neuroblastoma cell line (SH-SY5Y in hydrogel) alone and in co-culture with astrocytes (differentiated from human iPSCs), aiming for potential use in building the blood-brain barrier (BBB) *in vitro*. From the strain-stress curve, it was possible to obtain compressive modulus values in the order of 330 KPa and 110 KPa for GM7.5 and GM7.5_GX. The elastic modulus values (for 30% deformation) decreased by half in the composition with Geltrex, with values of 0.1 KPa, with low stiffness as expected in brain tissues (< 1 KPa). The scanning electron microscopy results showed about 50% porosity in both compositions. The pore size distribution also showed a higher frequency of pores in the 20-120 μ m range for both compositions. The softest composition was chosen for the viability assays (GM7.5_GX), monitored over 7 days using resazurin. The results showed that the cells remained viable during the analyzed period, with a large increase in metabolic activity compared to day 1. Furthermore, bright field microscopy showed that the cells presented morphology over the 7 days. These preliminary results show the potential of the composition (GM7.5_GX) from a mechanical and

biological point of view for BBB construction. Further experiments must be conducted by combining other cells of the BBB (endothelial cells) to investigate the viability and morphology of the co-culture on the microchips. In addition, permeability and immunofluorescence microscopy assays must be performed to characterize the model as a BBB.

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Modeling the porcine immune response to PCV2 infection

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Porcine Circovirus Type 2 (PCV2) is the primary causative agent of Porcine Circovirus Associated Disease (PCVAD), notably manifesting as Post-Weaning Multisystemic Wasting Syndrome (PMWS) in piglets, with mortality rates ranging from 4% to 30%. Due to its high transmissibility and the challenges of disease control on pig farms, PCV2 caused significant economic losses until the development of an effective vaccine in 2006.

In this study, we present a mathematical model for the porcine immune response to PCV2, formulated as a system of 13 ordinary differential equations and implemented in Python. Adapted from an existing model originally developed for simulating the human immune response to yellow fever, our model now describes the interactions between PCV2 viral particles and key cell populations within the porcine immune system – including antigen-presenting cells (Ap and Apm), lymphocytes (Thn, The, Tke, B, Bm, Ps, and PL), and antibodies (IgM and IgG). Leveraging the similarities between human and porcine immune systems, this model aims to enhance our understanding of PCV2 infection by facilitating the exploration of multiple infection scenarios and reducing reliance on animal experiments in line with the 3Rs principles to reduce, refine, and replace in animal testing.

The model was calibrated using experimental data on antibody concentrations and viremia in pigs at 21 days of age. Numerical simulations yielded time courses of viremia and antibody concentrations that qualitatively reproduced the expected trends of viral replication and immune response. Results indicate that viremia is primarily managed by the innate immune system during the first 10 days post-infection, after which the adaptive immune system elicits a delayed but sustained two-phase antibody response.

Future work will focus on refining the simulated outcomes and quantitatively validating them against literature data. Sensitivity analyses will be performed to explore diverse infection scenarios, and uncertainty quantification will assess the reliability of the model's predictions, thereby guiding further experimental validation. Overall, this approach aims to deepen our understanding of PCV2 infection and improve its management *in silico*, ultimately reducing the need for animal testing.

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Building knowledge maps to support NAM development

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Physiological maps (PMs) are comprehensive, machine-readable graphical representations of organ-specific biological mechanisms. These standardized frameworks integrate extensive knowledge from scientific literature to depict undisturbed physiology, creating valuable platforms for studying chemical-induced perturbations and their mechanisms. PMs streamline the integration, organization, and visualization of diverse biological, toxicological, chemical, and kinetic data to support New Approach Methodologies (NAMs).

Within ONTOX, five organ-specific PMs were developed: bile secretion & lipid metabolism (liver), nephron physiology (kidney), neural tube closure, and cognitive function development (brain). Their creation involved manual literature curation, ontological term annotation, and expert revision. PM development requires collaboration between domain experts and biocurators, guided by specific planning documents and curation guidelines with detailed metadata. This ensures compliance with FAIR principles (Findability, Accessibility, Interoperability, Reusability), facilitating use across projects and platforms. The modular structure simplifies review processes, enhances curation engagement, and enables complex data visualization for stakeholders.

Each map uses Systems Biology Graphical Notation (SBGN) for human interpretation, stored in machine-readable SBML format, and built using CellDesigner software following a workflow inspired by the Disease Maps Project. The MINERVA platform enables interactive exploration with a Google Maps-like interface, making complex biological information accessible.

These maps form the foundation for computational logic models that simulate system behaviors, contributing to ONTOX's *in silico* NAM toolbox. Future development will incorporate AI-assisted text-mining and automatic model updating to maintain current, human-relevant, and animal-free tools that serve as knowledge repositories. The PMs advance next-generation risk assessment by promoting collaboration between toxicology and systems biology communities, benchmarking adverse outcome pathways, identifying therapeutic targets, and visualizing omics data. Continuous refinement and collaborative development will enhance their utility in developing personalized medicine approaches and human-relevant risk assessment methodologies.

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Next generation risk assessment (NGRA) of hair dye HC Yellow No. 13: Ensuring protection from liver steatogenic effects

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This study employs animal-free NGRA principles to evaluate the safety of repeated dermal exposure to 2.5% (w/w) of the hair dye HC Yellow No. 13 (HCY13). As multiple *in silico* tools consistently flagged hepatotoxic potential, likely due to HCY13's trifluoromethyl group, which is known to interfere with hepatic lipid metabolism, liver steatosis was chosen as the primary mode of action for evaluation. Adverse outcome pathway (AOP)-guided *in vitro* tests were conducted, exposing human stem cell-derived hepatic cells to varying HCY13 concentrations over 72 hours. The expression of 11 lipid metabolism-related marker genes (AHR, PPARA, LXRA, APOB, ACOX1, CPT1A, FASN, SCD1, DGAT2, CD36, and PPARG) and triglyceride accumulation, a phenotypic hallmark of steatosis, were measured. PROAST software was used to calculate *in vitro* Points of Departure (PoDNAM) for each biomarker. Using GastroPlus 9.9, physiologically-based pharmacokinetic (PBPK) models estimated internal liver concentrations (C_{max} liver) of HCY13, ranging from 4 to 20 pM. All PoDNAM values significantly exceeded the predicted C_{max} liver, indicating that HCY13 at 2.5% (w/w) is unlikely to induce liver steatosis under the assumed conditions. This research demonstrates the utility of NGRA, integrating AOP-based *in vitro* assays and computational models to protect human health and support regulatory decision-making without animal testing.

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Automating the generation of QSAR prediction reporting format reports using OPERA models and KNIME

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Quantitative Structure-Activity Relationship (QSAR) models are central to modern toxicology, enabling rapid prediction of chemical hazards while reducing reliance on animal testing. The QSAR Prediction Reporting Format (QPRF) is a standard designed to serve as structured documentation of QSAR model predictions, providing information on model applicability and reliability for regulatory decision-making. These reports play a key role in ensuring transparency, reproducibility, and regulatory compliance in computational toxicology. However, the manual creation of QPRF reports remains a challenge due to its labor-intensive nature and susceptibility to human error. In response, we have developed an automated workflow using the Konstanz Information Miner (KNIME), an open-source data analytics platform, that harnesses QSAR predictions from the Open QSAR App (OPERA) to generate standardized QPRF reports efficiently.

The workflow begins with the direct import of output files from OPERA, which include a wide range of toxicological predictions such as physicochemical properties, environmental fate, and human health endpoints, along with detailed applicability domain assessments and confidence metrics. By leveraging KNIME's modular architecture, the system extracts, reformats and compiles this data into a structured document that complies with regulatory standards. This process significantly reduces manual data handling, ensuring consistency and enhancing transparency in report generation.

A key advantage of our approach is its integration into the Modeling and Visualization (MoVIZ) pipeline developed at the National Institute of Environmental Health Sciences (NIEHS). This integration allows users to transition seamlessly from model execution to report generation. Moreover, MoVIZ facilitates interactive data exploration, enabling users to examine trends, identify outliers, and validate model predictions before finalizing reports. Such features not only streamline the documentation process but also support more robust chemical hazard assessments by providing clear, reproducible insights into QSAR outputs.

Evaluation of our KNIME-based workflow indicates that it substantially reduces reporting time and improves the accuracy of QPRF documents compared to traditional manual methods. By automating the extraction and formatting of OPERA predictions, our solution enhances reproducibility and supports regulatory decision-making with consistent and transparent documentation. This workflow offers significant utility in computational toxicology by accelerating regulatory reviews and broadening the acceptance of QSAR-based assessments.

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Next generation risk assessment (NGRA): Elevating the risk assessment of cosmetics to new standards

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European Regulation No. 1223/2009 prohibits animal testing and marketing of animal-tested cosmetics since March 2013, creating as such a pressing need for developing animal-free methods, also called New Approach Methodologies (NAMs), to assess the safety of cosmetic ingredients. While validated NAMs exist for assessing local toxicity, there is a significant gap in methods for evaluating long-term systemic toxicity and developmental toxicity. These assessments are, however, essential for determining Point of Departure (PoD) values used for calculating the Margin of Safety (MoS) in quantitative risk assessment. NGRA offers a promising solution by utilizing an exposure-driven, tiered, and human-relevant framework that integrates *in chemico*, *in silico* and *in vitro* methods to support weight of evidence (WoE) safety decisions. Incorporating NGRA into regulatory decision-making could facilitate the introduction of safe new cosmetic ingredients (e.g., preservatives, UV-filters). The Scientific Committee on Consumer Safety (SCCS) has therefore incorporated NGRA into its Notes of Guidance, 12th Revision, and additionally created a safe harbor for submitting safety dossiers using NGRA alongside standard risk assessment methods. As such, the submission of a first dossier on the UV-filter Benzophenone-4 marked an interesting and valuable learning exercise for both the cosmetics industry and the SCCS. This process highlighted the need for developing a set of criteria for accepting not only validated NAMs but also “scientifically valid” NAMs within a WoE approach. Additionally, establishing acceptance criteria for parameter verification and analysis (sensitivity and uncertainty) for PBPK modelling is another barrier to overcome, in particular when applying *ab initio* NGRA. Typically, the ratio between simulated and observed kinetics data should meet certain criteria. However, since in case of *ab initio* NGRA no *in vivo* data are available, alternative parameters need to be developed and generally accepted to ensure reliability and regulatory approval.

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A weight of evidence approach to avoid animal testing in risk assessment of cosmetic ingredients – The SCCS perspective

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The Scientific Committee on Consumer Safety (SCCS) is a group of independent scientists that provides scientific advice to the European Commission on the safety of non-food consumer products and services, in particular cosmetics, regulated under Regulation (EU) N°1223/2009. This regulation mandates the SCCS to provide evidence-based safety assessments of cosmetic ingredients based on quantitative risk assessment-not hazard based evaluation/classification. Under a twin track system, the SCCS evaluates those substances for which concern exists for human health, while the Cosmetic Industry is responsible for assessing finished products and ingredients. Regulation (EU) N°1223/2009 is the first EU framework to completely ban animal testing and marketing of cosmetics, making the use of animal-free alternatives, known as New Approach Methodologies (NAMs), imperative. NAMs are key in the weight of evidence (WoE) approach applied by the SCCS for safety conclusions. As a single NAM is unlikely to provide sufficient evidence for safety, a WoE resulting from a combination of NAMs is generally required for safety assessments. Such WoE approach integrates human exposure information with various scientific data derived from *in silico* and *in chemico* methods, read-across, computational models, *in vitro* assays, defined approaches, new technologies (e.g., omics, ...), and historical *in vivo* data. This approach is considered strong when NAMs align with an Adverse Outcome Pathway (AOP) and address one or more of the Key Events (KEs). This tiered approach serves as a pioneering example of modern risk assessment, proving that reliable and high-quality safety evaluations can be conducted without animal testing. Nevertheless, also limitations and challenges are discussed, particularly concerning broader implications for human health.

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Advancing *in vitro* models for melanoma and glioblastoma research: A step toward animal-free systems

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Background: The development of robust *in vitro* models is crucial for reducing animal experiments and improving translational cancer research. Melanoma and glioblastoma remain highly challenging due to their complexity, heterogeneity, and limited treatment options. Current preclinical models often fail to replicate disease progression accurately, hindering effective therapeutic advancements.

Methods: For melanoma research, we established four novel cell lines (MUG Mel3) derived from a lymph node metastasis of a pregnant patient. These cell lines, cultured under different conditions, highlighting that hPL and FBS were used in comparison during cultivation exhibited distinct phenotypic, genotypic, and tumorigenic properties, providing an advanced system to study melanoma heterogeneity and pregnancy-associated tumor biology. Furthermore, synthetic human lactoferricin-derived peptides were tested successfully as a potential treatment strategy, particularly relevant for pregnancy-safe therapeutics.

In glioblastoma research, we successfully developed five continuously proliferating cell lines from ten primary glioblastoma patients, with four showing *in vivo* growth potential. Two recurrence-derived cell lines and a unique post-mortem glioblastoma model, established 40 hours after death, provide valuable insights into late-stage tumor progression. All cell lines underwent comprehensive characterization, including doubling time, CD133 expression, molecular profiling, mutation analysis, and comparison with parental tumor tissue.

Results and conclusion: Both model systems offer robust alternatives to traditional animal models by accurately replicating the complexity of melanoma and glioblastoma. The MUG Mel3 melanoma model allows drug, while post-mortem glioblastoma cell line MUG GB4PMenable a deeper understanding of terminal disease progression. These advancements pave the way for more precise, ethically responsible, and translationally relevant cancer research.

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Advancing toxicity predictions for chemical mixtures using artificial intelligence: *Raphidocelis subcapitata* as a model organism

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Industrial chemicals, including pesticides, pose significant risks to living organisms. Research indicates the toxicological hazards associated with these substances, particularly their mixtures – combinations of non-chemically bonded components. Given that chemical exposure often involves multiple agents, experimental testing for these mixtures is impractical due to the wide variability of components and their concentrations. To address this challenge, *in silico* methods have been developed to predict toxicity. The combined effects of chemicals can be conceptualized through two models: Independent Action (IA) and Concentration Addition (CA) [1]. The aim of this study is to develop predictive Quantitative Structure-Toxicity Relationship (QSTR) models for assessing the toxicity of chemical mixtures using computational methods, specifically focusing on the algae *Raphidocelis subcapitata*, a species recommended for ecotoxicological bioassays by OECD (2011).

We retrieved biological assay data for compounds involving mixtures of *R. subcapitata* from various sources, including the Chemical Hazards Database, Tox21, EPA, ECHA, and relevant literature. The data preparation process followed the methodology outlined by Cherkasov et al. [2] Standardization of the data was performed using RDKit in Python to minimize common errors found in publicly available databases. Utilizing embeddings from Roberta and ChemBERTa [3,4], we constructed a chemical space that classifies mixtures into CA

and IA categories based on structural similarity, allowing for inference of shared mechanisms. Additionally, we assessed the discontinuity in biological data to evaluate how mixture concentrations influence biological responses, thereby providing a deeper understanding of the relationship between chemical structure, biological activity, and variability in effects [5]. The mixture data extracted from biological assays in scientific literature, though limited, formed the basis for developing models, totaling 107 chemical mixtures tested against *R. subcapitata*. The analysis of chemical space enabled the successful classification of 97 chemical structures into CA and IA, with only a few molecules presenting classification inconsistencies, likely due to the limitations of the representation methods used. Looking ahead, the generated embeddings will not only aid in constructing the chemical space but will also serve as descriptors in predictive modeling using classical machine learning algorithms, such as Support Vector Machines (SVM), Random Forest, LightGBM, and XGBoost. We will also explore advanced approaches, including graph-based models and Chemical Language Models, to enhance the vector representation of molecules and improve the predictive capabilities of our models. Validation will be conducted with rigorous statistical methods to ensure the accuracy and reliability of the classifications achieved.

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Dermal absorption profile of agrochemical formulations using pig skin tissues

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Although a waiver may be considered, the dermal absorption study is a mandatory requirement for agrochemical registration in Brazil, as determined by RDC 294/2019. While excised human skin is considered the gold standard, its limited availability and use in Brazil has led to the consideration of pig skin as a locally available alternative. Although pig skin is generally accepted as a reasonable surrogate for human skin in dermal absorption studies, this assumption is primarily based on cosmetic products evaluations, with limited research conducted on agrochemicals. This study aims to address this gap by comparing the dermal absorption profiles of different active ingredients (AI) present in commercial agrochemical formulations. Radiolabeled active ingredients were tested as concentrated and one dilution in two commercial formulations. The selected agrochemicals are both emulsifiable concentrate (EC) formulations with different active ingredients and distinct log Pow values. The two ingredients have distinct physicochemical properties, with Ingredient 1 in Formulation A having a log Pow of -1.2 and Ingredient 2 in Formulation B having a log Pow of 2.9. Given that a higher Log Pow value indicates greater lipophilicity, it is anticipated that Formulation B would exhibit a higher dermal absorption profile compared to Formulation A. This research shall not only provide valuable insights into the comparative dermal absorption profiles of these formulations using different dermal absorption models but also contribute to the broader understanding of using pig skin as a surrogate for human skin in agrochemical dermal absorption studies. These findings have significant implications for agrochemical assessments, particularly when alternatives could be required for evaluating human health risks. Moreover, data obtained using alternative methods may inform the development of criteria for potential waivers in future regulatory frameworks, streamlining the assessment process while maintaining safety standards.

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From rabbits to reconstructed human epidermis: A shift in skin irritation testing for agrochemical formulations

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Agrochemical formulations necessitate rigorous testing for hazard classification and labeling. Traditionally, the Draize rabbit skin test (OECD 404) has been the standard method for assessing skin corrosion and irritation, despite concerns regarding its relevance to human health protection, reproducibility, as well as ethical implications regarding animal welfare. Two *in vitro* OECD test guidelines utilizing reconstructed human epidermis (RhE) have been validated to predict skin irritation and corrosion potential: OECD 431 (RhE-Cor), which differentiates corrosive from non-corrosive substances, and OECD 439 (RhE-Irr), which identifies irritating from mildly/non-irritating substances. However, these tests have not been validated for complex mixtures such as agrochemicals.

This study aims to assess the effectiveness of *in vitro* RhE assays and the GHS concentration threshold (CT) method for determining skin irritation and corrosion hazard classifications in agrochemical formulations. A retrospective analysis was conducted, comparing the concordance of classifications derived from *in vitro* tests and the CT approach with available *in vivo* data for 187 formulations from CropLife Brasil companies. Based on historical *in vivo* data provided for these formulations, 18 (10%) were classified as Category 2, 29 (16%) as Category 3, and 140 (75%) as Non-classified (NC) under GHS criteria. There were no Category 1 formulations represented in the data set. Considering the *in vitro* data available from these formulations, 35 were tested solely by RhE-Cor, 73 were tested solely by RhE-Irr, and 54 were tested by both methods.

The results indicated that for formulations assessed solely through RhE-Cor using the “top-down” approach (Cat. 1 vs. Cat. 2/3/NC), 94% (33/35) were classified as true negatives. In contrast, the “bottom-up” approach (Cat. 1/2 vs. Cat. 3/NC) for formulations assessed solely for RhE-Irr yielded an 81% (59/73) concordance rate, with a negative predictivity (NPV) of 95%. When both RhE-Cor and RhE-Irr were utilized,



the “top-down” concordance rate was 93% with a 100% NPV, while the “bottom-up” concordance rate was 59%, with an NPV of 89%. These findings suggest that RhE-Irr may be sufficient for assessing agrochemicals, given that the majority of formulations are not classified for skin irritation under GHS criteria.

The CT approach demonstrated a “top-down” concordance rate of 97% with a 100% NPV, while the “bottom-up” concordance rate was 74%, with a 95% NPV. These results highlight the potential to reduce animal testing through possible test waivers and provide a rationale for addressing data gaps in skin irritation assessments. Given that the vast majority of agrochemical formulations are not classified for skin irritation under GHS criteria, as supported by the data set of the present study, it is highly likely that RhE-Irr and the CT approach reliably predict formulations that do not require classification for skin hazard potential.

In conclusion, although the analyses do not entirely eliminate the necessity for animal testing in the evaluations of skin irritation for agrochemical formulations, they indicate a pathway for reducing animal use by utilizing non-animal methods.

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Evaluation of biofilm formation in tridimensional ex vivo models of reconstructed human skin and spheroid infected with *T. rubrum* and analysis of associated virulence genes

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Introduction: *Trichophyton rubrum* is a keratinophilic fungus, one of the most important dermatophytosis agents. This fungus presents various virulence factors, and biofilm formation is one of them, but few studies have explored this aspect. *In vitro* experimentation cannot accurately study interactions with the host. Recently, skin equivalents

have been increasingly studied for their ability to simulate *in vivo* conditions, but the need remains to explore it with biofilm formation. In this study, the infection of 3D reconstructed human skin and spheroids was evaluated, as well as biofilm formation, its interactions with the models, and the expression of associated genes.

Objectives: To employ alternative *ex vivo* models of 3D reconstructed human skin (RHS) and spheroid to evaluate *T. rubrum* infection and biofilm formation with the expression of genes associated with biofilm formation and their relationship with pathogenesis.

Methods: *Trichophyton rubrum* ATCC-MYA 4438 was cultured in planktonic form for 7 days, with biofilm formation after 72 hours. Fungal biomass and metabolic activity were assessed using crystal violet and tetrazolium salt reduction-XTT tests. The 3D RHS model was assembled from primary cultures of keratinocytes and fibroblasts and spheroid formation using human keratinocyte cells (HaCaT). Both models were infected with a 1×10^6 CFU/mL conidia solution. After spheroid formation in 96 h, fluorescence microscopy, and sphericity index were employed, and both infected models were analyzed using confocal laser scanning microscopy-CLSM and scanning electron microscopy-SEM. The total RNA of infected RHS was extracted and quantified, and its purity was assessed using the 260/280 absorbance. RNA was treated with DNase I to remove genomic DNA and subsequently converted into complementary DNA (cDNA). qPCR was performed using fungalsin (MEP5) and subtilisin (SBT5) genes associated with dermatophyte pathogenesis, with GAPDH as the endogenous reference gene. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Results: *T. rubrum* biofilm reached maturation after 72 hours, characterized by high metabolic activity and biomass production. Similar structures were observed in both the *in vitro* biofilm and the RHS infection model, suggesting biofilm formation occurred during its infection. 3.0×10^4 and 6.0×10^4 cells/well were optimal for spheroid formation. CLSM analysis revealed keratinocyte survival throughout the experiments and the fungus' complete colonization of the spheroid surface. SEM analysis demonstrated the formation of characteristic fungal structures indicative of biofilm development during the infection of both 3D models. Gene expression analysis using qPCR revealed an increased expression of metalloprotease-5 (MEP-5) during the infection of the reconstructed three-dimensional skin model.

Conclusions: This study can potentially clarify new perspectives about dermatophyte biofilms. Both three-dimensional infection models demonstrated a good capability for *T. rubrum* development with characteristics compatible with biofilm formation, indicating its importance in host-fungus interaction. The dermatophyte's high expression of the MEP-5 gene presents itself as a possible marker or target for drug development.

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AI-driven predictive models for identifying endocrine-disrupting chemicals targeting the progesterone receptor

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The progesterone receptor (PR) plays a critical role in regulating essential biological functions, including reproduction, metabolism, and development. Environmental exposure to endocrine-disrupting chemicals (EDCs) that interfere with PR signaling is linked to adverse health outcomes, such as metabolic disorders, reproductive challenges, and hormone-related cancers. To address the urgent need for efficient identification of PR-targeting EDCs, this study leverages advanced artificial intelligence (AI) tools to develop predictive models that reduce reliance on animal testing. Using a quantitative structure-activity relationship (QSAR) framework, we integrated molecular structural data with biological activity profiles to create robust *in silico* models. A rigorously curated dataset of PR-interacting compounds was analyzed through chemical space mapping via t-SNE and enriched with natural language processing (NLP) to decode activity trends. The AI models achieved high predictive accuracy: for PR agonists, balanced accuracy (BACC) was 0.82 (sensitivity [Se] = 0.79, specificity [Sp] = 0.83), while antagonists yielded a BACC of 0.81 (Se = 0.80, Sp = 0.82), with Matthews correlation coefficients of 0.56 and 0.57, respectively. These results validate the models' ability to distinguish active from inactive compounds reliably. To maximize accessibility, these tools are being optimized for deployment on the InsightAI platform (<http://insightai.labmol.com.br>), enabling researchers and industry stakeholders to screen chemicals for PR disruption rapidly. This work directly supports the "3Rs" principles – reducing, refining, and replacing animal use in toxicology – by offering a computational alternative to traditional *in vivo* assays. This project exemplifies the transformative potential of AI in advancing ethical science. By prioritizing *in silico* methods, we contribute to global efforts to minimize animal experimentation while accelerating the identification of hazardous chemicals, thereby safeguarding public health and ecosystems.

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WAT-liver-on-chip: Bridging adipose and hepatic insights into insulin resistance

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Multi-organ chips (MOCs) are an emerging technology that provides a species-specific platform well suited to study metabolism by recreating organ-level function, making them a valuable tool for studying the dynamic crosstalk between organs [1]. Insulin resistance is a well-characterized metabolic disorder, particularly regarding its clinical manifestations and downstream effects. However, the precise molecular mechanisms driving insulin resistance remain incompletely understood, largely due to the limitations of existing human-relevant disease models. Here, we simulate the dynamic crosstalk between liver and adipose tissue with the use of a PDMS-free vascularized WAT-Liver chip, as a relevant human microphysiological *in vitro* model for studying insulin signaling. Human subcutaneous white adipocytes, human microvascular endothelial cells and stromal vascular fraction (SVF) cells were isolated from skin biopsies for the white adipose tissue (WAT)-chip while the HepaRG[®] cell line was used as a liver model for simulating WAT-Liver crosstalk. Characterization of the vascularized WAT-liver chip showed that it maintained adipocyte morphology and unilocularity, preserved endothelial CD31 expression, and supported the formation of a continuous endothelial monolayer covering both tissues under fluidic flow [2]. HepaRG cells were Albumin, MRP-2 and E-cadherin positive in the co-culture medium and when connected downstream of the WAT-chip. Time-resolved insulin response using healthy post-prandial (fed) glucose and insulin levels showed lipolysis inhibition, increase in lactate production, significant FOXO1 translocation from nuclei to cytoplasm, and translocation of GLUT4 to the cell membrane. Single organs and 2-organ communication were also evaluated by measuring fatty acid content in the liver, effluent analysis glycerol and triglyceride production by the WAT and liver respectively and by response to glucose/insulin exposure (11mM and 10-6000 nM, respectively). Additional single-cell RNA sequencing and targeted gene expression analysis of the white adipose tissue revealed cellular heterogeneity and transcriptional responses. Finally, to improve data interpretation, equivalent data is collected from WAT-chips, and their secretome is analyzed to characterize donor-specific metabolic responses. Experimental results



from WAT and Liver-WAT chips are integrated with publicly available metabolic databases. This integration enables comparative analysis and identification of key regulatory pathways between the studied tissues, and predictive modeling of metabolic outcomes.

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Making an adsorbent from fish scales for adsorption of azo dyes from industries

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Show how animals and animal by products solves environmental problems.

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Making “doing the right thing” even easier – The harmonization of the monocyte-activation test (MAT) for the testing of inherently pyrogenic vaccines at Sanofi

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To deliver safe products to patients, and as part of regulatory requirements, vaccine manufacturers must demonstrate that their products contain acceptable levels of pyrogens. Pyrogens are currently detected using rabbits that are inoculated with products in the “Rabbit Pyrogen Test” (RPT). To reduce or eliminate the use of these animals, we have developed and qualified the Monocyte-Activation Test (MAT) for testing our drug products for pyrogenicity. The MAT is an *in vitro* assay that detects the presence of pyrogens in a sample by incubating it with human-derived monocytes. If exogenous pyrogens are present, the monocytes are stimulated to produce cytokines. The measurement of the cytokine response to a sample is then compared to controls and is used as a readout to assess the pyrogen content. The European Pharmacopoeia currently endorses two MAT methods, Method 1 and Method 2. Presented here is a comprehensive MAT method for analyzing multiple Sanofi Drug Product (DP) vaccines. This assay was qualified for precision, repeatability, linearity, and specificity (including the detection of endotoxin and non-endotoxin spikes). The implementation of this assay for testing Sanofi’s vaccine products will allow for the monitoring of safety and consistency without the use of rabbits which is in keeping with our company’s adherence to the 3Rs principle.

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All authors are employed by Sanofi and may hold shares and/or stock options in the company.

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Brazil's regulatory landscape for veterinary products: Challenges and opportunities in adopting alternative methods to animal testing

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Brazil has yet to establish clear regulatory acceptance of alternative methods for registering veterinary products, in contrast to global advancements in ethical and scientific approaches to product evaluation. Traditionally, the safety and efficacy of these products have been assessed through *in vivo* studies involving species-specific animals such as dogs, cats, and calves. However, international regulatory agencies have increasingly promoted the adoption of alternative methods aligned with the principles of Replacement, Reduction, and Refinement (3Rs), aiming to minimize animal testing while maintaining scientific rigor. While the European Union, the United States, and several Asian countries have made significant progress in integrating alternative models into their regulatory frameworks, Brazil is still in the process of reevaluating its approach. The European Union has been a leader in implementing alternative methods, requiring that all possible non-animal testing options be considered before resorting to *in vivo* studies. Institutions such as the European Centre for the Validation of Alternative Methods (ECVAM) play a crucial role in promoting and validating these approaches. Similarly, the United States Food and Drug Admin-

istration (FDA) has launched the New Alternative Methods Program, aiming to accelerate the adoption of non-animal methods that improve predictivity, assess toxicological risks, and streamline the development of FDA-regulated products. The FDA acknowledges that these methods can provide valuable insights into disease mechanisms and enhance safety evaluations without reliance on traditional animal testing. In Asia, countries such as Japan and South Korea have increasingly engaged in initiatives to harmonize their regulations with international standards. Collaborative efforts with global organizations have helped advance the acceptance of alternative methods, ensuring their regulatory frameworks remain aligned with evolving scientific developments. Brazil is also moving toward regulatory discussions on alternative methods. Since 2023, legislative reviews and public consultations related to veterinary products have been underway to assess the feasibility of integrating non-animal testing approaches into the registration process for veterinary products. While this process is still ongoing, it reflects a broader effort to align with global advancements in ethical research practices and regulatory science. International organizations such as the World Organization for Animal Health and the Organization for Economic Co-operation and Development (OECD) continue to play a key role in facilitating the adoption of alternative methods. The OECD regularly updates its testing guidelines to incorporate validated non-animal approaches, enabling greater international harmonization of product assessments, including the possibility of using for veterinary products. As regulatory frameworks worldwide continue evolving, the integration of alternative methods presents an opportunity for greater scientific innovation and alignment with ethical research practices. Countries at different stages of adoption contribute to a diverse global landscape, where ongoing advancements and policy developments shape the future of veterinary product evaluation.

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Colorectal cancer-on-a-chip: A biomimetic *in vitro* model to advance basic and translational research

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Introduction: Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies, ranking third among all tumors and being the second leading cause of cancer-related death worldwide. Its progression and treatment response are strongly influenced by tumor-microenvironment interactions. Adherens junction markers, such as E-cadherin, play a crucial role in assessing treatment response and disease prognosis. Traditionally, animal models are used to study CRC and evaluate therapies. However, ethical concerns and translational limitations arise, as these models often fail to accurately mimic the human tumor microenvironment, compromising the clinical relevance of preclinical findings. In this context, Organ-on-a-Chip (OoC) technology emerges as an innovative alternative for *in vitro* modeling of the tumor microenvironment, providing an advanced biomimetic system that reduces reliance on animal models. This approach enables the co-culture of healthy and tumor human cells in a relevant 3D environment, facilitating studies on tumor resistance and drug screening, with greater translational potential.

Objective: To develop a 3D biomimetic *in vitro* model of the CRC microenvironment using Organ-on-a-Chip (OoC) technology for studies on tumor progression and therapeutic resistance mechanisms.

Methods: To establish co-cultures within the device, endothelial cells (EA-hy926) and mesenchymal cells (Hs-5) were incorporated into a collagen hydrogel, representing the lamina propria, and deposited into the compartments of the device developed by our group. After collagen solidification, type IV collagen was added to mimic the basement membrane, followed by the deposition of epithelial cells (Caco-2 C2BBel), which were incubated for 48 hours. To simulate pathological conditions, intestinal tumor cells (HCT116) were subsequently seeded onto the epithelial layer. The analyses performed included: (i) assessment of cell viability using trypan blue exclusion and live/dead staining with calcein and propidium iodide via confocal microscopy; (ii) evaluation of tissue architecture through histological analysis (H&E staining); and (iii) detection of adherens junction protein E-cadherin expression by immunofluorescence.

Results: Our results demonstrated that the device was capable of maintaining cell viability over 24, 48, 72, and 96 hours of co-culture, as evidenced by trypan blue staining and calcein labeling. Additionally, the spatial distribution of cells was preserved within the designed geometry. The model also enabled a detailed evaluation of tissue organization, resembling that found in the human intestine, as confirmed by histological analysis. Notably, the system recapitulated E-cadherin expression after 48 hours, a crucial protein in tumor progression, suggesting the organotypic functionality of the model.

Conclusion: Collectively, our findings suggest that the OoC platform can overcome the limitations of animal models by accurately mimicking

the tumor microenvironment and enabling detailed analyses of colorectal cancer progression and therapy response.

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Standardization of a repetitive magnetic stimulation (rMS) protocol in neuroblastoma cell lines: Preliminary results

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Considering the epidemiological, social, and economic impact, cancer can be regarded as one of the most complex public health concerns. It represents a significant percentage of death causes, and despite recent pharmacological advancements, current treatments available may cause severe side effects and poor quality of life. In this context, neuromodulatory techniques (electrical or electromagnetic) can be employed to modulate specific neural activities within the central nervous system (CNS). These interventions are considered safe, however, the mechanism of action and possible side effects are mostly misunderstood.

Currently, repetitive magnetic stimulation (rMS) is used as an alternative or complementary method to treat some neurological conditions, including chronic pain and major depression. Previous studies conducted by our research group, using static magnetic stimulation (sMS) in neuroblastoma cells (SH-SY5Y), demonstrated a line-specific reduction of cellular proliferation and viability, suggesting that magnetic stimulation may engage as an adjuvant therapy for CNS tumors.

Therefore, this experimental study was designed to standardize a method of rMS in cell culture by exposing a tumor cell line to this stimulatory method. Stimulation was delivered via a magnetic cell stimulator developed by the Biomedical Engineering team at the Hospital de Clínicas de Porto Alegre (INPI-ID: BR20201502878[®]). The device was designed and impressed in a 3D FDM Ultimaker 3 (Wishbox Technologies[®]) to couple the cell culture plate and cover it with a controlled magnetic field (comprising 6 coils, 300mT $\pm$ 2% of magnetic field intensity). For this purpose, neuroblastoma cells were allocated to two distinct groups: SHAM rMS and active rMS. Cells were exposed to a daily session of SHAM or active rMS (300mT/1Hz) in different periods (75, 150 e 300 seconds) for 4 consecutive days.

Cellular viability and cell death were evaluated using Trypan Blue count exclusion and ANNEXIN/Pi assay, respectively. Contrasting the reported sMS results, the viability and number of death cells of stimulated tumoral cells were similar between SHAM and active groups, demonstrating the safety and possibility of rMS application in cellular culture. This protocol will be repeated to increase the experimental "n" and evaluate the reproducibility of the findings. Furthermore, possible genes and protein-specific alterations resulting from this intervention will be assessed in future studies. Our results reinforce the safety of rMS and indicate the need to develop more extended exposure time protocols to detect potential anti-tumoral effects.

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DRIVES: Developing resources for *in vitro* evidence synthesis

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In vitro preclinical research plays a crucial role in biomedical research; it is essential for our understanding of human health and disease-related pathophysiology, and for the drug development pipeline. At the same time, it complements *in vivo* and clinical studies permitting detailed approaches to unravel mechanistic phenomena that can be further applied in the refinement and development of alternatives to animal-based research. Albeit its central role in the translational pathway, the synthesis of evidence from *in vitro* studies has proven problematic. Human screening of *in vitro* publication titles and abstracts missed detecting 14% of publications [1], as *in vitro* methods and results are often not summarized. Moreover, methodological and

quality features of *in vitro* studies were missing overall with some features only reported in 15-30% of the studies [2]. This inconsistency of reporting reduces the possibilities to synthesize evidence from *in vitro* studies that could potentially inform 3R approaches, which are on the rise given their priority to replace and reduce animal studies. Considering this, the DRIVES project aims to create an infrastructure, following rigorous practices, that facilitates the synthesis of *in vitro* research. For this purpose, we will define the aspects of *in vitro* methods that threaten their internal and experimental validity to incorporate them in a tool to appraise studies for evidence synthesis. In addition, we will collate published literature with high relevance for human health that contains *in vitro* methods to ease the systematic identification of these studies. Finally, we will assess the current reporting and methodological practices in *in vitro* evidence synthesis with the goal to develop guidance for the planning and registration of systematic reviews of *in vitro* studies. With these objectives, we foresee the inception of a standard approach to synthesize and appraise *in vitro* literature in a meaningful way for the preclinical and clinical fields.

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Development of an *ex vivo* model for assessing pulmonary toxicity mechanisms

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Introduction: Inhalation of toxic substances can lead to airway irritation, inflammation, and sensitization. There is a need to explore new approach methodologies (NAMs) that replicate the human respiratory system. Human *ex vivo* models, such as Precision Cut Lung Slices (PCLS), simulate the pulmonary cellular environment with functional cells and extracellular matrix, providing a more accurate representation of lung physiology. However, the limited availability of human tissue is an obstacle to the widespread use of these models. The porcine PCLS (pPCLS), due to its similarity to the human lung, emerges as a promising alternative model, offering great potential for early evaluations. These pPCLS are also promising for cryopreservation strategies, enabling prolonged sample storage. Additionally, innovative techniques such as air-liquid interface (ALI) culture, microphysiological systems (MPS), and aerosol exposure systems can be applied, mimicking real-life exposure scenarios.

Objective: To develop and characterize an *ex vivo* pPCLS model to assess pulmonary toxicity mechanisms.

Methodology: pPCLS were prepared using a Tissue Slicer DTK-3000W and maintained at an ALI for twenty days. For cryopreservation assessment, selected samples were preserved for one year using either a chemically defined freezing medium or a solution composed of culture medium and DMSO. Additionally, pPCLS were cultured in the MPS, both under submerged conditions and at an ALI, for twenty days. Acute and repeated exposures were conducted on pPCLS, which were exposed to aerosolized flame retardant tributyl phosphate using the Vitrocell® Cloud 12 exposure chamber. Tissue viability was assessed, and morphological analysis was performed. After processing, histological examination was conducted using hematoxylin-eosin and Mallory staining. Markers of reactive oxygen species (ROS) and mitochondrial activity, as well as MUC5AC and active Caspase-3, were evaluated by indirect immunofluorescence. Additionally, molecular mechanisms involving K-RAS, TNF- α , Ki67, and cleaved Caspase-3 were analyzed using the western blot technique.

Results and discussion: The pPCLS in ALI maintained tissue viability and alveolar morphology for twenty days. However, morphological differences were observed between static and dynamic models, as well as statistical differences in inflammatory markers, suggesting that culture in microphysiological systems can influence the long-term maintenance of these models. Furthermore, differences in morphology and tissue viability were observed between the chemically defined freezing medium

and the solution composed of culture medium and DMSO, indicating that the model can be cryopreserved in a chemically defined freezing medium for up to one year. Additionally, following exposure to aerosolized known toxicant, in this case, flame retardant tributyl phosphate, an increase in ROS and MUC5AC, with decrease in mitochondrial activity, and activation of caspase 3 were observed. Additionally, the modulation of K-RAS, TNF- α , Ki67, as well as cleaved caspase-3, were observed demonstrating that the model is promising for evaluating and elucidating the mechanisms of pulmonary toxicity.

Conclusion: The pPCLS model holds significant promise as a valuable tool for pulmonary toxicity assessment, providing insights into mechanistic processes and advancing our understanding of lung toxicity, in particular for flame retardants.

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BINACLE assay for *in vitro* detection of tetanus neurotoxin: Optimization perspectives for quality control use

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In light of its commitment to 3Rs and its ambition to fully replace *in vivo* analytical testing, our company has been taking part in the EDQM BSP 136 collaborative study since its early phases. This collaborative study aims at validating a functional assay which uses the specific receptor-binding and proteolytic properties of tetanus neurotoxin, the BINACLE (binding and cleavage) assay for the detection of tetanus toxicity in toxoids in order to implement an *in vitro* alternative to the current guinea-pig model for the detection of active tetanus toxin. In parallel, with the aim of introducing a method fully applicable to an industrial QC environment and fully in line with our 3Rs policy, our analytical development laboratory has been performing optimizations on the assay notably regarding the use of alternatives to critical animal derived reagents and chemical reagents with occupational safety concern as well as reducing assay cycle time. Our presentation will therefore describe the optimizations already performed or ongoing along with comparative results obtained on different matrices. Lastly, we will open on remaining challenges for next steps to further develop and implement this assay in collaboration with EDQM and other stakeholders.

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Emmanuelle Coppens is a Sanofi employee and may hold shares and/or stock options in the company.

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Organ-on-a-chip technology for leukemia: A step towards reducing and replacing animal testing

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Introduction: Leukemia is the most prevalent cancer in children, with the bone marrow microenvironment playing a crucial role in disease progression and treatment response. Chemotherapy remains the primary therapeutic approach; however, treatment resistance, short remission duration, and mortality persist in many cases. Moreover, toxicity, adverse effects, and high hospitalization costs underscore the need for novel therapeutic strategies. In drug development, preclinical models are essential for guiding clinical trials. However, conventional 2D cultures fail to replicate key aspects of the dynamic *in vivo* microenvironment, while animal models, despite providing a complete cellular context, raise ethical concerns and exhibit interspecies differences in pharmacokinetics, pharmacodynamics, immune responses, and tissue-organ interactions, limiting their translational relevance. In this context, Organ-on-a-Chip (OoC) platforms emerge as promising alternatives, enabling physiologically relevant *in vitro* models.

Objectives: This study aims to establish an OoC-based model that recapitulates the bone marrow microenvironment under healthy and leukemic conditions.

Methods: A computationally designed OoC device was fabricated using polydimethylsiloxane (PDMS), incorporating lateral channels representing blood vessels and trabecular bone, along with a central chamber mimicking the medullary niche. Numerical simulations assessed fluid velocity profile and oxygen distribution within the system. For cell culture, endothelial cells (EA-hy926) were seeded in the vascular channel, while osteoblasts (Saos2) were cultured in the endosteal channel. To model healthy and leukemic conditions, mesenchymal stromal cells (Hs5) alone or co-cultured with leukemic cells (B-ALL REH) were embedded in a hydrogel simulating the extracellular matrix (ECM) and introduced into the central chamber. The system was maintained under continuous fluid flow for 72 hours and analyzed for cell viability (Trypan Blue), niche formation (CellTracker labeling), and proliferation (Ki67 staining).

Results: Numerical simulations demonstrated homogeneous interstitial fluid flow with physiologically relevant velocities and the formation of an oxygen gradient akin to *in vivo* conditions. Viability assays confirmed the maintenance of co-cultures for 72 hours. Confocal microscopy revealed the formation of an endothelial barrier resembling vascular walls and a trabecular bone-like osteoblast layer. In the central niche, mesenchymal and leukemic cells interacted with vascular and trabecular structures. Leukemia progression was evidenced by tumor cell proliferation and increased niche cell proliferation in the presence of leukemic cells.

Conclusions: These findings indicate that the OoC platform successfully mimics critical aspects of the leukemia bone marrow microenvironment. This model holds potential for reducing animal experimentation, accelerating anti-leukemic drug discovery, and advancing the understanding of tumor resistance mechanisms.

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Effects and search for peptides from the immune response of *Galleria mellonella* under different conditions of *Cryptococcus neoformans* infection

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Studies of human pathologies are essential for the identification of therapeutic targets and the development of new treatments. One of the central issues in this research is the choice of experimental models, considering the ethical and scientific challenges in the use of animals. As an alternative, *in vivo* models with alternative animals, such as *Galleria mellonella*, have gained prominence due to their cellular and biological complexity, which is increasingly being investigated, as well as metabolic pathways that mimic aspects relevant to the study of diseases. The animal also possesses molecules described as potential tools against pathogens. The larval stage of *G. mellonella* is widely used to investigate immune responses, especially those mediated by hemocytes and antimicrobial peptides, which are considered part of the insect's humoral response. Additionally, the animal is a well-established model for studies of fungus-host interactions, being applied to evaluate the pathogenesis of microorganisms such as *Cryptococcus neoformans*. Research has already demonstrated that this model can elucidate fungal virulence factors and aspects of the insect's innate immunity. This study proposes the use of a technique classically employed for mice, "epitope masking," to investigate the immune response of *G. mellonella* when infected with *C. neoformans* with masked epitopes, comparing it to the response to unmasked cells. The experiments involved electrophoretic separation and purification of antimicrobial peptides by chromatography, followed by their biological characterization against the microorganism used as the immunizing agent. The antimicrobial activity was evaluated in relation to the overall aspect of hemolymph from immunized larvae under the established conditions. The results showed that *G. mellonella* is an effective model for assessing the plasticity of the immune system in response to antigen masking, contributing to the understanding of complex immune responses. The technique revealed changes in the production of antimicrobial polypeptides under masking conditions, pointing to potential biomarkers and new therapeutic targets. Thus, the study reinforces the role of *G. mellonella* as a versatile and ethical tool for immunological and pathogenic research.

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Physiologically based kinetic (PBK) modeling-facilitated reverse dosimetry to estimate N-nitrosodimethylamine (NDMA) point of departure

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N-Nitrosodimethylamine (NDMA), a potent carcinogen, has emerged as a critical concern in the pharmaceutical industry due to its presence as an impurity in drug products. Regulatory agencies worldwide, including the US FDA, ANVISA (Brazil) and EMA, have set strict limits on NDMA exposure, with current regulatory assessments relying on animal-derived cancer potency estimates. The established point of departure (PoD) for NDMA, based on rodent bioassays, is 95,900 ng/kg/day. However, there is an urgent need to refine nitrosamine assessments using new approach methodologies (NAMs) to improve human relevance and reduce reliance on animal data. This study presents an initial step toward refining NDMA risk assessment using a physiologically based kinetic modeling-facilitated reverse dosimetry (PBK-RD) approach to estimate a data-driven PoD, as a model to proof-of-concept for derivation of new limits for nitrosamines. As a first phase, a PBK model for NDMA was developed in rats using PK-Sim version 11.3, incorporating NDMA physicochemical properties and rat species-specific parameters and clearance pathways. Model performance was verified by comparing simulated and observed plasma pharmacokinetic (PK) profiles from NDMA intravenous doses, with predicted parameters falling within an acceptable range (0.5- to 2-fold) of observed data, in line with OECD (2021) guidelines. To derive a PoD, *in vitro* toxicity data from rat hepatocyte cell models were analyzed, focusing on NDMA-induced DNA damage and cytotoxicity. Benchmark dose modeling software (BMD v3.3.2, US EPA) was used to determine the lower 5% benchmark dose (BMDL5). The validated PBPK-RD model of NDMA in rats was then applied to translate *in vitro* concentration-response data into *in vivo* dose-response. The NDMA PBK-RD-derived PoD was compared to the current regulatory PoD, highlighting potential refinements in risk assessment. The next steps of this study include validating the PBK model for NDMA oral doses, incorporating the liver as the target organ for toxicity, and extrapolating the model to humans by applying species-specific parameters during allometric scaling. This study demonstrates the utility of PBK-RD as a NAM-based approach to improve NDMA risk assessment in the pharmaceutical industry, supporting a more mechanistically informed and human-relevant derivation of new nitrosamines safety limits without animal tests.

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Ending the LD50 test: 49 years and counting

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Opposition to the LD50 test began around fifty years ago. In 1979, the UK Home Office produced a report on the test recommending its continuation after inviting comment from industry, academe and animal welfare organizations. Opposition to the test was considerably strengthened following critical reports of the LD50 by practicing toxicologists in 1968 (JK Morrison et al.) and 1981 (G Zbinden & Flury-Roversi). However, the LD50 test was firmly embedded in chemical safety assessment, and the test was not easy to replace as documented in a 1988 ATLA Editorial by Michael Balls. In the USA, animal activist, Henry Spira campaigned against the test and managed to extract an admission in the late 1980s from David Rall, a former Director of the National Toxicology Program, that the LD50 test “was an anachronism.” Nevertheless, the test was embedded in regulatory language in many countries and continued to be performed to satisfy box-ticking requirements. Recently, a computer modelling system to estimate toxicity has been developed by ICCVAM in the US. This approach may finally offer a non-animal alternative to the various lethal dose tests that are still being performed today.

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Cellular lesion quantification to enhance the classification of eye irritants using the bovine corneal opacity and permeability (BCOP) test

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Currently, TG 492B OECD is the only officially recognized animal-free *in vitro* method capable of distinguishing between ocular irritants and chemicals that induce severe eye damage, according to the UN-GHS classification (category 2 for irritants and category 1 for severe

irritants). Additionally, the OECD Guideline 467 provides Defined Approaches for assessing potential hazards based on the UN-GHS classification for serious eye damage and irritation.

On the other hand, the BCOP test (OECD TG 437) is a validated methodology for identifying chemicals that induce serious eye damage and those that do not require classification for eye irritation or serious eye damage. It has been suggested that histological studies could complement the BCOP test by providing additional insight into the irritation potential of a substance.

Histologically, the cornea consists of three layers: epithelium, stroma, and endothelium. The quantification of lesions occurring in these structures during BCOP assay exposure may provide further information on mild or moderate irritants.

In this study, three different SDS (sodium dodecyl sulfate) concentrations were selected based on *in vivo* Draize test results, where they were reported as mild (1% SDS), moderate (3% SDS), and severe (10% SDS) irritants. The BCOP test was used to estimate their irritation potential, finding that SDS 1% was unclassifiable, while for SDS 3% and 10%, no standalone prediction could be made.

To identify variables that could aid in classifying different irritation levels, opacity, permeability, and histological lesions (quantitative or semi-quantitative) were assessed in the epithelium (intraepithelial edema, spongiosis, desquamation, and chromatin condensation), and endothelium (presence or absence). Groups of six corneas were exposed to three SDS concentrations (1%, 3%, and 10%) and incubated for 10 and 30 minutes.

Histological changes were evaluated using H&E staining at 20X magnification. In the epithelium, affected cells were counted, while changes in the stroma (edema) were graded as mild, moderate, or severe, and changes in the endothelium were classified as present or absent. A 3×2 factorial design was applied, followed by Kruskal-Wallis and Dunn’s post hoc analysis to determine which variables contributed to a more detailed classification.

Among the analyzed variables, permeability (in corneas incubated for 10 and 30 minutes) and intraepithelial edema (in corneas incubated for 30 minutes) revealed significant differences among SDS concentrations. However, changes in the stroma and endothelium did not contribute to the classification.

Counting intraepithelial edema-affected cells in bovine corneas exposed to different SDS concentrations allowed differentiation between mild and moderate irritation. Therefore, quantifying histological findings in the epithelium of bovine corneas in the BCOP assay could help refine the classification of mild and moderate irritants.

Additionally, extending the incubation time beyond the standard BCOP test conditions (from 10 to 30 minutes) may improve the visualization of epithelial corneal lesions. Future studies should expand the analysis to include other types of substances, including additional surfactants.

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h-VIOS: A human-relevant drug discovery and development platform using bioprinted tissues

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Over the past two decades, several innovations have significantly enhanced our ability to predict the safety and efficacy of therapeutics in humans, with the overarching goal of reducing failure rates and expediting the translation of these therapies to the clinic. These advances span a range of enabling tools, including bioprinting and tissue engineering technologies, *in vitro* models such as spheroids, organoids, and organ-chips, and *in silico* strategies that employ machine learning alongside large chemical, biological, and clinical datasets. Despite these advancements, a persistent challenge has been the generation of human-relevant, reproducible preclinical data at scale. Traditional animal models often yield false positives and negatives due to intrinsic differences from human pathophysiology, while overly simplistic *in vitro* models struggle to capture the complexity of human biological processes crucial for assessing therapeutic safety and efficacy. The h-VIOS™ platform addresses this challenge by integrating bioprinted organ-chips that enable therapeutic delivery through endothelialized vasculatures into complex, three-dimensional tissues. This modular and customizable system enables the generation of multimodal data, ranging from high-content imaging to transcriptomics. In this presentation, we will explore the h-VIOS platform and discuss its applications in contexts-of-use such as drug-induced liver injury (DILI), drug-induced vascular injury (DIVI) prediction, and the evaluation of on-target, off-tumor effects of anticancer therapeutics.

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Fetal bovine serum composition: A barrier to standardization in cell-based assays?

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The transition toward animal-free science presents significant challenges, particularly in replacing fetal bovine serum (FBS) in cell cultures. Despite its widespread use, FBS introduces methodological barriers due to its variable composition and the risk of microbiological contamination. Therefore, identifying alternative solutions which align with the principles of the 3Rs (Reduction, Refinement, and Replacement) is essential to ensure reproducible assays and more predictive methodologies. The replacement of FBS relies on robust and complementary data, enabling the adoption of more complex evaluation systems in a synergistic and predictive manner.

This study aimed to analyze the qualitative and quantitative composition of different FBS samples, focusing on the quantification of growth factors, vitamins, hormones, lipids, proteins, ions, organic compounds, and the presence of Mycoplasma contamination was assessed.

Three FBS samples from different regions (Brazil, the United States, and Paraguay) were analyzed before and after heat inactivation at 56°C for 30 minutes. Mycoplasma contamination was qualitatively assessed using the MycoAlert™ PLUS Mycoplasma Detection Kit, while biochemical components and growth factors were quantified. A total of 58 biochemical parameters were evaluated, and specific growth factors (EGF, IGF-1, bFGF, and VEGF-A) were measured by ELISA following lyophilization.

The results showed that none of the analyzed samples were contaminated with Mycoplasma. However, among the 58 biochemical parameters evaluated, 20 exhibited variations ranging from 16% to 102% (Folic Acid, Adrenocorticotrophic Hormone, Anti-thyroglobulin Antibody, Direct Bilirubin, Indirect Bilirubin, Iron Binding Capacity – Latent, Estradiol, Alkaline Phosphatase, GAMMA GT, LDL, Luteinizing Hormone, Parathyroid Hormone, Total T3, Glutamic Pyruvic Transaminase, Transferrin, Vitamin C) in non-inactivated samples, while 19 parameters varied between 16% and 84% (Folic Acid, Amylase, Anti-Thyroglobulin Antibody, Direct Bilirubin, Iron Fixing Capacity Latent, Estradiol, Alkaline Phosphatase, Gamma-Glutamyl Transpeptidase, Insulin, Lactate Dehydrogenase, Luteinizing Hormone, Parathyroid Hormone, Glutamic Pyruvic Transaminase, Glutamic Oxaloacetic Transaminase, Transferrin, Triglycerides, Vitamin C, Vitamin D3) after heat inactivation. Among the growth factors analyzed, only bFGF and VEGF were detectable by ELISA. In contrast, EGF and IGF-1 were below the detection limits of the method (0.82 pg/mL and 1,229 ng/mL, respectively). Besides, heat inactivation impacted significantly in growth factor concentrations of the samples.

These findings reinforce that variability in FBS composition can impact the reproducibility of *in vitro* assays. Therefore, identifying and quantifying FBS components is crucial to understanding their influence on cell culture. However, to establish culture systems entirely free of animal-derived components, further research is needed to develop efficient universal substitutes that enhance predictability and scientific reliability.

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New approach methodologies for photogenotoxicity: Filling the gaps in photosafety testing

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The current battery of photosafety testing primarily assesses photoreactivity and *in vitro* photocytotoxicity, with the 3T3 Neutral Red Uptake (NRU) and Reconstructed Human Epidermis assays as the only validated methods. However, these approaches do not assess photogenotoxicity, as they are restricted to detecting photocytotoxicity. While adaptations of genotoxicity assays under UV-vis exposure exist, they remain non-validated, with inherent biomodel limitations. Additionally, *in silico* toxicology is widely used for hazard assessment, but available photosafety models are based on the 3T3 NRU assay, restricting their ability to predict photogenotoxicity reliably. Evidence shows that some compounds yield negative results in standard *in vitro* and *in silico* tests while exhibiting photomutagenic effects. In this context, integrating New Approach Methodologies (NAMs) – combining *in vitro* and *in silico* techniques – offers a promising strategy to enhance phototoxicity and genotoxicity assessments. This study applied an integrated approach to evaluate the mutagenic and phototoxic potential of a selected class of substances – agrochemicals – using an alternative yeast-based *in vitro* model (*S. cerevisiae* yno1 strain) alongside expert rule-based and statistical *in silico* predictions. While none of the tested substances exhibited mutagenic potential, glyphosate and 2,4-D herbicides demonstrated photomutagenicity in the yeast model, despite negative *in silico* phototoxicity predictions and negative results in the *in vitro* 3T3 NRU phototoxicity test. These findings highlight a critical gap in the current photosafety evaluation framework, emphasizing the need to incorporate complementary photogenotoxicity assays. Expanding the existing test battery with robust *in vitro* and *in silico* methodologies is crucial to preventing false negatives and improving the overall safety assessment of chemical compounds.

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How institutional support can accelerate the advancement of new approach methodologies (NAMs) in biomedical research – Key findings from the BioMed21 workshop in Brazil

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The biomedical research landscape is undergoing significant transformation, with increasing global investment in the development of New Approach Methodologies (NAMs). These innovative technologies – including organoids, organs-on-chips, and artificial intelligence – have the potential to drive major advancements in biomedical research. NAMs address critical challenges in drug development and biomedical research, such as the need for human-relevant models that better replicate human biology than traditional methods, the advancement of personalized medicine, the reduction of high drug attrition rates, and the ethical concerns surrounding animal testing.

To address these challenges, Humane World for Animals organized a workshop in March 2025 in Brasília, Brazil, to explore how institutional support can accelerate the adoption of NAMs in the country. The event gathered Brazilian researchers specializing in NAMs, funding agencies, and government representatives to develop a coordinated strategy for increasing investment in this field.

This presentation summarizes the key outcomes of the workshop, outlining a roadmap for advancing biomedical research centered on human biology in Brazil. Key elements include:

- Intersectoral collaboration among academia, industry, regulatory agencies, and international partners,
- Regulatory acceptance and global standardization of new technologies,
- Sustainable, long-term funding models, and
- Education and training programs to build an interdisciplinary workforce.

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Nano-bio interactions: Impacts of *Escherichia coli* biocorona formation on biological assays with nano-materials and the *Caenorhabditis elegans* model

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Caenorhabditis elegans is a well-established alternative model organism widely applied in human and environmental toxicology research. Its diet consists mainly of bacteria, which serve as its primary source of nutrients. Under experimental conditions, *Escherichia coli* is typically provided as food to prevent *C. elegans* starvation. However, the influence of *E. coli* biocorona formation over the biological assays performed with nanomaterials has often been overlooked. This study evaluated the impact of *E. coli* biocorona formation on the properties, behavior and toxicity of graphene oxide (GO), both alone or in combination with silver ions (Ag⁺). Our results reveal that *E. coli* biomolecules coated the GO surface, altering its thickness, surface roughness, composition, charge and colloidal behavior. *E. coli* biocorona also modified GO toxicity, alleviating its adverse effects on nematode survival, neurons, germline cells, and the intestinal barrier. On the other hand, when combined with Ag⁺, *E. coli* biocorona significantly increased Ag⁺ lethality to *C. elegans* by 4.6 times. Confocal Raman spectroscopy revealed that biocorona-coated GO combined with Ag⁺ was translocated to secondary organs of *C. elegans*, where it induced apoptotic corpses in germline cells and caused neuronal damage. Indeed, *E. coli* biocorona acted as a carrier agent of silver since nematodes accumulated two times as much silver as those exposed to uncoated GO combined with Ag⁺. Our findings provide the first evidence of the dual role of the *E. coli* biocorona in modulating GO toxicity. This emphasizes the need for the *C. elegans* research community to consider the biocorona effect and nano-bio interactions as critical factors in nanosafety evaluations. Neglecting the effects of biocorona not only increases the risk of generating inconsistent findings but may also compromise the reliability of *C. elegans* as an alternative model organism for nanotoxicology, raising concerns about its relevance and translational applicability for human health risk assessment.

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Evaluation of an adapted agar diffusion cytotoxicity assay for oncological medical devices using the HepG2 cell line

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Medical devices are designed for human use in the prevention, diagnosis, treatment, and management of diseases. Their primary goal is to enhance health and quality of life. Biomaterials used in these devices span various fields, including biomedical engineering. Due to the specific nature of these products, animal models are often required for efficacy and safety assessments, as traditional monolayer cell cultures present limitations. The agar diffusion cytotoxicity assay is widely employed to assess the safety of medical devices by evaluating material biocompatibility. However, we identified an internal demand for an efficacy test of a novel medical device intended for hepatocarcinoma treatment via obstruction and drug lixiviation mechanisms. Given the limitations of monolayer cell cultures for this specific application, we adapted the agar diffusion cytotoxicity assay using the HepG2 adenocarcinoma cell line to evaluate its suitability for oncological medical devices.

HepG2 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin. The cells were maintained in 75 cm² flasks at 37°C in a 5% CO₂/95% air atmosphere with controlled humidity. After cell counting, suspensions were prepared at the following densities: 1×10⁵ cells/mL (for 24- and 48-hour readings), 5×10⁴ cells/mL (for 72- and 96-hour readings), and 2×10⁴ cells/mL (for 120- and 144-hour readings). The suspensions were plated in 6-well plates (2 mL/well). After 24 hours, 1.8% agar in twice-concentrated culture medium (1:1) was added. Once solidified, filter paper disks containing the test sample, positive control (2% sodium dodecyl sulfate, SDS), and negative control (PBS) were placed on the agar. To assess cell viability, an MTT solution (0.5 mg/mL) was added after the exposure periods, and halo formation was evaluated after 3 hours.

For the positive control, the average diameters of the cell death halos were: 11 mm (24 hours), 11.67 mm (48 hours), 12 mm (72 hours), 12-13 mm (96 hours), 13-14 mm (120 hours), and 12.33 mm (144 hours). The progressive increase in halo size over time confirmed the method's effectiveness in detecting material diffusion and cytotoxic effects. In contrast, no halo formation was observed for the negative control.

Following method standardization, we concluded that the diffusion process enables a progressive evaluation of cell death via MTT reduction assay at different cell concentrations. Cell plating at 1×10⁵ and 5×10⁴ cells/mL, with exposure times of 48 and 72 hours, respectively, allowed for a uniform assessment of cell viability beyond the specimen exposure. The formation of a cell death halo around the specimen, along with viable cells beyond the halo, demonstrated the method's applicability for evaluating drug diffusion from oncological medical devices.

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A novel *in vitro* approach to understanding odor perception and aging for inclusive fragrance design

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Odor detection, a complex process involving human olfactory receptors (hORs) in the olfactory epithelium and subsequent processing in the brain, plays a crucial role in various aspects of human experience. About 400 hORs have been identified, enabling the discrimination of several hundreds of thousands or more. However, the intricacies of hOR activation and their impact on odor perception remain largely unexplored.

Our partner ChemCom has developed an innovative *in vitro* testing system encompassing a library of more than 700 cloned sequences corresponding to all known functional hORs genes and frequent genetic variants, that can be expressed in cells. This platform includes the 270 ORs that were shown to be present in the human olfactory epithelium, 90 of which are common to all individuals. This technology utilizes HEK293T-RTP1S/RTP2 cells (licensed by Duke University) designed to express the different receptors whose activation can be detected through a luciferase-based bioluminescence assay. The high-throughput capacity of this system allows for the screening of thousands of samples weekly.

In our study, we analyzed 19 fragrance samples with known olfactory profiles and age-related acceptance data to explore the relationship between hOR activation and fragrance preference across different age groups. Our goal was to investigate how different fragrance profiles activate hORs and if this activation could be correlated with acceptance by different age groups.

Initial findings, with 48 different ORs activated by all 19 fragrances, highlighted the complexity of fragrance perception. Some receptors (OR7A17, OR1A1, and OR7C1) were activated by all tested fragrances, while OR2J2 and OR5A1, for example, were activated by one fragrance. Further research is needed to establish clear connections between specific receptor activation profiles and fragrance characteristics. Future studies may involve increasing the number of fragrances tested and/or using pure ingredients instead of complex fragrance mixtures.

This *in vitro* platform allows the study of olfactory receptors in a high-throughput format without needing to rely on animal models or invasive human testing. A larger study can further support the understanding of consumer perception and explore possible relations between olfactory discomfort and specific molecules.

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Dual application of bovine corneal hydrogel in 3D reconstructed cornea and acellular models for ocular irritant identification

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Ocular toxicity assessment is essential for evaluating the safety of chemicals, pharmaceuticals, and cosmetic products while promoting the reduction of animal testing. Conventional methods such as the Draize test have been widely criticized due to ethical concerns and interspecies variability. To address these limitations, innovative *in vitro* models using biomimetic materials have been developed. This study investigates the application of bovine corneal extracellular matrix (ECM)-derived hydrogel in two alternative *in vitro* models: a 3D reconstructed corneal epithelium model and an acellular assay based on ECM-protein interactions. The 3D model was developed using a decellularized corneal ECM hydrogel scaffold, supporting the growth and stratification of human corneal epithelial cells. This model mimicked the human corneal epithelium and allowed the evaluation of cellular responses to chemical exposure. The acellular assay utilized ECM hydrogel to assess protein coagulation and changes in turbidity, providing a rapid and cost-effective pre-screening method for potential ocular irritants. The methodologies were applied to 22 substances classified according to the Globally Harmonized System (GHS). The acellular model exhibited high specificity (100%) in correctly identifying non-irritants; however, its sensitivity (30%) for irritant detection was limited, indicating a need for further optimization. The 3D model effectively distinguished irritants from non-irritants, with histological analyses revealing maintained epithelial integrity in non-irritant exposures and cellular degeneration in irritant conditions. The integration of these methods offers a comprehensive, ethical, and sustainable approach to ocular toxicity assessment. These findings contribute to the refinement of *in vitro* models for regulatory applications and support the transition toward animal-free testing strategies in toxicology.

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Assessing alternative methods in preclinical research: The AMRL framework

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Preclinical research is essential for developing new therapies, yet it faces increasing pressure to align scientific progress with ethical and responsible practices, especially regarding the use of animal biomodels. The Alternative Methods Readiness Level (AMRL) was developed as an innovative management tool, adapted from the Technology Readiness Level (TRL) framework, which classifies technological maturity on a scale from 1 to 9. AMRL assesses the readiness of alternative methods to animal testing, supporting the transition to more ethical and effective approaches aligned with the 3Rs principles (Replacement, Reduction, and Refinement). The AMRL framework consists of 76 targeted questions, covering critical scientific, technical, and regulatory aspects necessary for the validation and implementation of alternative methods. By offering a structured assessment, AMRL enables researchers, industry professionals, and policymakers to make data-driven decisions about adopting alternative models. To validate its applicability, AMRL was tested in two distinct case studies:

SkinEthic™ RHE – TRL9 – This reconstructed human epidermis model is widely used for toxicological and dermatological testing. Classified at TRL9, SkinEthic™ RHE is fully developed, validated, and ready for regulatory and industrial adoption. Its evaluation through AMRL confirms the tool's ability to identify mature alternatives to traditional animal testing.

Monocyte Activation Test (MAT) – TRL3 – The MAT is an alternative to the rabbit pyrogen test for detecting fever-inducing contaminants. Classified at TRL3, it remains in an early developmental stage, requiring further validation. This case highlights AMRL's capacity to assess diverse technologies at different maturity levels, offering insights into the necessary steps for regulatory approval and broader adoption.

The results demonstrate that AMRL is an effective tool for evaluating and advancing alternative methods, helping stakeholders navigate the complex landscape of preclinical research innovation. By providing a clear readiness metric, AMRL facilitates informed decision-making, accelerates innovation adoption, and reinforces adherence to the 3Rs principles. We believe that AMRL marks a major step toward a more sustainable, ethical, and scientifically robust future in preclinical research. It bridges the gap between innovation and application, ensuring that scientific progress and animal welfare evolve hand in hand.

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Tiered approach to develop an animal-free 3D bioimprinted epidermal model for safety assessment

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The development of alternative methods to animal testing was a groundbreaking achievement in the life sciences. It addressed both ethical and scientific concerns, accelerating progress at an unprecedented pace and reducing costs. However, many of these methods still rely heavily on animal-derived components. Besides ethical concerns, their use also raises questions about the relevance of results in studies focusing on human health. Replacing such components with human-derived or chemically defined alternatives is crucial. Therefore, this project aimed to evaluate alternatives to animal-derived components used in the development of 3D reconstructed human epidermis (RHE) skin models. Our objective was to develop a relevant skin model for use in safety assessment tests, such as skin irritation (OECD 439). Commercially available animal-free keratinocytes were utilized to develop RHE skin models using both manual and bioprinting techniques. For comparison, RHE models using animal-derived components were also produced manually and through bioprinting. Morphological analysis was performed using skin biomarkers through immunofluorescence to compare the "animal-derived components" and "animal-free" conditions, as well as the manually and bioprinted models. Furthermore, tests utilizing the positive and negative control substances outlined in OECD 439 were evaluated on the epidermis models under all conditions to assess similarities in toxicity response and ensure that differences in the reconstruction process and components used did not affect physiology or response mode. These results will pave the way for developing a more accurate model that can fully replace the use of animal-derived components in the reconstruction of 3D models used for safety assessments.

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Development of a Brazilian organ-on-a-chip technology for bioinspired vascular engineering in diabetic wounds and reduction of animal use in preclinical studies

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Diabetes Mellitus (DM) is a major global health challenge, affecting 8.8% of adults worldwide. Projections indicate that its prevalence may exceed 642 million cases by 2040. Among its complications, diabetic foot ulcers (DFUs) represent a critical public health issue, associated with high morbidity, recurrent infections, and increased amputation risk. Current therapeutic approaches are often insufficient, as they fail to effectively address impaired angiogenesis, chronic inflammation, and tissue regeneration deficits.

Angiogenesis is essential for wound healing, yet it is severely compromised in diabetic patients due to endothelial dysfunction, oxidative stress, and persistent inflammation. Traditional models, including 2D cell cultures and animal models, fail to accurately replicate the diabetic microenvironment, necessitating innovative alternatives. Advances in bioengineering have led to the development of Organ-on-a-Chip (OoC) platforms, which provide biomimetic *in vitro* environments to study pathophysiological mechanisms and test novel therapeutics.

CHIP-Eny is an advanced microfluidic OoC platform designed to replicate the microenvironment of DFUs with high fidelity. Developed through a collaboration between the University of Brasília (UnB) and the National Center for Energy and Materials (CNPEM), CHIP-Eny integrates mathematical modeling, computational simulations, and 3D bioprinting to optimize experimental conditions. Computational Fluid Dynamics (CFD) analyses and structural simulations were employed to ensure mechanical integrity, optimize fluid flow, and enhance endothelial cell responses under controlled conditions.

The platform consists of biomimetic microfluidic channels that recreate vascular architecture, facilitating interactions between endothelial cells, extracellular matrix components, and controlled biochemical gradients. It enables precise replication of pathophysiological conditions such as hypoxia, oxidative stress, and inflammation, improving translational research potential.

Unlike conventional *in vitro* models, CHIP-Eny is compatible with various microfluidic peristaltic pumps, ensuring accessibility and versatility for multiple applications. The platform supports angiogenesis studies, medical device testing, and pharmaceutical screening, offering a physiologically relevant alternative to animal models. Initial validation studies have confirmed its effectiveness in replicating key vascularization processes and providing reproducible results in wound healing research.

Adherence to the 3Rs principles (Replacement, Reduction, Refinement) positions CHIP-Eny as a technological innovation in biomedical research, promoting the reduction of reliance on animal experimentation. Its large-scale implementation, particularly in Brazil's Unified Health System (SUS), has the potential to improve reproducibility, reduce costs, and expand access to advanced *in vitro* models. Although the technology still requires further testing for its definitive adoption, its concept already significantly contributes to regenerative medicine and personalized therapies.

This technology is protected under Patent pending (BR 20 2025 004913 4), ensuring intellectual property rights and supporting its future applications in biomedical engineering and clinical research.

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Organ-on-a-chip platforms as an alternative for *in vitro* translational research in regenerative medicine

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Translational research aims to apply scientific advances to improve patient care, following a well-established sequence of stages: T0 (basic research), T1 (preclinical studies), T2 (clinical trials), T3 (clinical implementation), and T4 (impact on population health). Traditionally, translational research relies on preclinical models like 2D cell cultures and animal testing, but these methods struggle to replicate the complexity of human environments, which can lead to translational failures and limitations in clinical applications.

Microfluidic platforms, such as Organ-on-a-Chip (OoC) systems, have emerged as a promising alternative to overcome these limitations. OoC technologies provide biomimetic *in vitro* models that replicate human tissue physiological conditions with greater accuracy, offering a more relevant and ethical solution for translational research.

An innovative example of these platforms is the CHIP-Eny, developed by researchers from the University of Brasília and the National Center for Energy and Materials (CNPEM). The CHIP-Eny integrates computational modeling, fluid dynamics simulations, and 3D bioprinting to enhance experimental reproducibility and clinical translation,

with a specific focus on biomimetic simulation of chronic wounds, such as those associated with diabetic foot disease.

In preclinical research, these platforms allow therapy and intervention screening under physiological conditions without the need for animal models, which not only reduces the number of animals used in experiments but also improves reproducibility and the relevance of results.

In the translational research phase (T2), these platforms can be used to test pharmacological therapies and medical devices under conditions that are closer to physiological ones, enabling real-time evaluation of treatment effects on biological processes such as angiogenesis and inflammation in chronic wounds. This facilitates the transition from laboratory discoveries to clinical trials (T3), where microfluidic platforms, such as the CHIP-Eny, can align with regulatory standards from AN-VISA and FDA, supporting their adoption as alternative models for drug screening and medical device evaluation.

At the implementation stage (T4), OoC platforms, like the CHIP-Eny, can be integrated into healthcare systems, such as Brazil's Unified Health System (SUS), facilitating access to innovative and personalized treatments. The ability of these platforms to be produced with additive manufacturing and customized to meet individual clinical needs also reduces experimental costs and accelerates the development of new drugs, improving access to advanced biomedical technologies and positively impacting population health.

Microfluidic platforms align with the ethical principles of the 3Rs (Replacement, Reduction, Refinement). With their high reproducibility, human relevance, and potential to accelerate the approval of innovative therapies, these platforms represent a great promise for precision medicine, regenerative medicine, and the more efficient transition of scientific discoveries into actual patient care. The CHIP-Eny, with its integration of different technologies, is at the forefront of this innovation, providing a high-quality solution to address the challenges of modern translational research.

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In vivo oxidative stress assessment of polymeric nanoparticles containing amphotericin B using the zebrafish (*Danio rerio*) model

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Amphotericin B (AmB) is a drug used to treat various diseases, such as cutaneous and visceral leishmaniasis; however, its primary application is against fungal infections. The treatment is administered intravenously, requiring patient monitoring due to its dose-dependent toxicity and acute infusion-related reactions. One approach science employ to develop new AmB formulations, aiming to reduce adverse effects and enhance bioavailability, is nanotechnology. Polymeric nanoparticles (PNPs) are nanomaterials widely used in amphotericin B research. In this study, poly(lactic acid) (PLA), known for its high biocompatibility and degradation via simple hydrolysis, and polycaprolactone (PCL), which offers controlled degradability and high biocompatibility, were chosen to nanoencapsulate this drug. The zebrafish (*Danio rerio*) model is extensively used due to its rapid response time in analyses, greater sustainability, and compliance with Brazilian legal guidelines for animal testing models. This model is advantageous in oxidative stress studies because larval immersion enables high absorption rates and effective toxicological evaluations. This study aimed to assess the occurrence of oxidative stress in zebrafish (*Danio rerio*) larvae following exposure to polymeric nanoparticles containing Amphotericin B. The results indicate AmB toxicity, showing high levels of malondialdehyde and significantly reduced concentrations of reduced glutathione and catalase compared to the control group. Treatments with PNPs without AmB did not exhibit variations in toxicity. The findings from PCL PNPs with AmB suggest a detoxifying action, potentially mediated by osteogenesis-related transport pathways, along with increased production of reactive oxygen species (ROS) and varying biomarker responses. On the other hand, PLA PNPs with AmB demonstrated a possible diffusion of ROS and oxidative stress biomarkers throughout the organism, resulting in less dose- and time-dependent variation. Further specific studies are necessary to better understand the action and transport pathways of PLA. In conclusion, there is no significant difference in ROS concentration between the selected PNPs for AmB delivery. However, evaluating these nanoparticles' distinct mechanisms of action is essential.

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Computational modeling and systems biology for predictive preclinical studies: The case of CHIP-Eny

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Computational modeling and systems biology (SB) have revolutionized preclinical research by providing predictive frameworks that improve experimental design, reduce costs, and optimize therapeutic interventions. Traditional translational research heavily relies on *in vivo* models, which often exhibit species-dependent variability and limited applicability to human physiology.

The integration of Computational Fluid Dynamics (CFD), finite element analysis (FEA), and multi-scale biological modeling enables the simulation of complex biological processes, enhancing the accuracy of *in vitro*-to-*in vivo* correlations.

Among these applications, vascular modeling plays a critical role in regenerative medicine, particularly in conditions like diabetic foot ulcers (DFUs), where angiogenesis is impaired. CHIP-Eny, an advanced Organ-on-a-Chip (OoC) platform, Patent pending BR 20 2025 004913 4, an innovation developed by the University of Brasília in collaboration with the National Center for Research in Energy and Materials (CN-PEM), was analyzed to evaluate its effectiveness in replicating human-like vascularization dynamics. The study incorporated CFD-based shear stress analysis, endothelial response prediction, and dynamic culture conditions, allowing for the precise replication of angiogenic pathways in diabetic-like microbial environments.

Mathematical Bond Graph modeling was used to predict endothelial cell proliferation and angiogenesis behavior in diabetic capillaries, with data collected from the literature. Structural simulations validated the platform's mechanical stability, ensuring that flow rates and pressure gradients replicated physiological conditions. Experimental data from CHIP-Eny demonstrated that angiogenic sprouting and cellular responses were correlated with model predictions, highlighting its potential for preclinical drug screening and biomarker validation.

The importance of incorporating these tests, not only in Organ-on-a-Chip (OoC) but also in a broader range of alternative and complementary models, is crucial for the advancement of biomedical research. In particular, the integration of microfluidic platforms like CHIP-Eny can expand experimental possibilities in a scenario that more accurately simulates human conditions. These platforms, when combined with other advanced methodologies such as computational modeling and bioinformatics techniques, allow for a more comprehensive evaluation of therapies, treatments, and medical devices under controlled, yet representative, biological conditions.

Furthermore, transitioning from conventional *in vivo* models to approaches based on OoC and other microphysiological alternatives can be seen as a significant contribution to research ethics. This aligns with the principles of the 3Rs (Replacement, Reduction, Refinement), which aim



to reduce the reliance on animal testing by substituting them with systems that more faithfully replicate biological processes. This approach can not only improve the reproducibility of results but also accelerate the validation and approval of new therapies without the limitations and costs associated with animal testing.

Therefore, the OoC platform, when combined with these new computational models, not only opens new possibilities for preclinical research but also has the potential to accelerate the transition to clinical practice, directly impacting precision and regenerative medicine.

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“Nose to brain 3D”: Development of a 3D nasal model in microphysiological systems for the investigation of neurotoxicity through inhalation

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Recently, the atmospheric concentration of particulate matter (PM) has become a crucial factor in pollution assessment, considering its risks to public health. In this context, the olfactory pathway is particularly relevant via, allowing pollutants to rapidly reach the central nervous system, impacting brain function and structure. This has been explored in R&D in drug delivery for targeting neuronal tissues to treat diseases. However, despite its importance, the nose-to-brain pathway remains poorly studied, particularly regarding exposure to environmental pollutants like PM_{2.5} ($\leq 2.5 \mu\text{m}$). Additionally, most research prioritizes systemic effects, such as cardiovascular and respiratory outcomes, while neurological effects receive less attention. Thus, further investigations are needed to understand the mechanisms by which PM_{2.5} and other pollutants access the neurological tissue by the nose-to-brain via. In this context, the present study aimed to develop and characterize a 3D nasal epithelium model to be integrated into a microphysiological system associated with brain organoids exposed to aerosol of MP. For this purpose, the nasal epithelial cell line RPMI 2650 was cultured at an air-liquid interface for 21 days in transwell inserts with $0.4 \mu\text{m}$ pores. The nasal epithelium barrier function was assessed through transepithelial electrical resistance measurement and FITC-Dextran permeability assay. Histological analysis, using hematoxylin and eosin staining, confirmed the formation of a cohesive epithelium. Immunophenotypic characterization evaluated the presence of biomarkers E-cadherin, ZO-1, Occludin, Claudin-2, α -tubulin, MUC-5AC, and MUC-1, confirming that the developed tissue represents a nasal epithelium. Further studies will utilize nasal tissue combined with a 3D brain organoid model integrated into a microphysiological system to investigate the effects of PM_{2.5}, exposure via inhalation, on neuronal tissue.

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Toxicological evaluation of micro-emulsion-gel of yellow passion fruit seed oil (*Passiflora edulis* f. *flavicarpa*) in zebrafish (*Danio rerio*) embryos

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The number of cosmetic and pharmaceutical products derived from natural sources has increased in recent decades, with fruits being particularly prominent. Various parts of fruits can be utilized, including the peel, pulp, and seeds. Yellow passion fruit seed oil (PSO) exhibits promising skin-healing properties due to its high fatty acid content. Microemulsion-gels combine the characteristics of hydrogels with nanocarriers, providing unique efficacy. This study assessed the toxicity of a microemulsion-gel formulation containing PSO, compared to a formulation without oil, at different concentrations (96 $\mu\text{g/mL}$, 240 $\mu\text{g/mL}$, 600 $\mu\text{g/mL}$, 1500 $\mu\text{g/mL}$, and 3750 $\mu\text{g/mL}$), using the Fish Embryo Toxicity Test (FET) in accordance with OECD guideline 236. Toxicity testing using zebrafish (*Danio rerio*) has gained increasing recognition as an alternative model to mammalian testing due to the numerous advantages offered by this species. In recent years, the development of alternative methods has become a priority, and the Fish Embryo Acute Toxicity Test (FET), based on zebrafish embryos, has emerged as a replacement for acute toxicity testing in adult fish, aligning with the 3Rs principle to minimize animal use. Parameters such as embryo coagulation, number of deformities, body length, eye diameter, and heart rate were evaluated. The results indicated significant changes in the toxicity score of both formulations at concentrations of 600 $\mu\text{g/mL}$ and 1500 $\mu\text{g/mL}$, with a higher number of deformities. Regarding body length, the only significant change occurred with the white gel at 240 $\mu\text{g/mL}$, where larvae exhibited greater length than the control group. For eye diameter, significant changes were observed at the 240 $\mu\text{g/mL}$ concentration of both the white and PSO gel. Regarding heart rate, a significant alteration was seen only with the PSO gel at 240 $\mu\text{g/mL}$. The 600 $\mu\text{g/mL}$ and 1500 $\mu\text{g/mL}$ concentrations did not provide a valid sample size for other evaluations beyond deformity scoring. The observed changes at the higher concentrations suggest that while PSO may hold promise for cosmetic applications, further evaluation is essential to determine the safe and effective concentration for human use. The study also highlights the importance of the zebrafish toxicity model for screening cosmetic formulations, enabling a detailed analysis of adverse reactions across various physiological parameters.

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Toxicological assessment of sugars found in children's beverages

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Introduction: The use of sugars in children's beverages has become an increasing concern due to their impact on children's health. These frequently consumed beverages contain significant amounts of added sugars, which can lead to problems such as obesity, dental cavities, and metabolic diseases. Early and frequent exposure to these sugars may affect child development and establish harmful eating habits. Therefore, it is essential to study the toxicity of the sugars present in these beverages to better understand their long-term effects and develop effective strategies to protect children's health.

Objective: To analyze the toxicological impact of sugars found in children's beverages on zebrafish (*Danio rerio*) embryos and larvae.

Methodology: The study was conducted following the guidelines of OECD guide 236. In summary, embryos exposed to glucose, fructose, and sucrose samples were evaluated over 96 hours, at 24-hour intervals, alongside a negative control group. At the end of the exposure period, images were taken for morphological evaluation, and 10-second videos were recorded to count each fish's heartbeats with the aid of a counter. Using the images, the body length (from head to tail) and eye diameter of each animal were measured. To select the tested concentrations, successive dilutions were performed based on the concentrations found in children's beverages.

Results and discussion: The concentrations of sugars tested, as found in children's beverages, proved lethal to the embryos. In the case of sucrose, the second-highest concentration also showed toxicity, reaching near-total lethality, while glucose and fructose had similar results in their dilutions. For body length, eye diameter, and heart rate measurements, only concentrations with more than 50% of live animals per replicate were considered. Regarding body development, the 0.0518 g/mL concentration of sucrose was the only one that differed from the negative control, indicating that the larvae grew less than expected, suggesting potential growth retardation. No differences were observed in heartbeats per minute.

Conclusion: The sugar concentrations found in children's beverages were harmful to zebrafish embryos and larvae, causing not only mortality at higher concentrations but also growth retardation.

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Advancing diabetic microvascular dynamics prediction with CHIP-Eny: Mathematical modeling and control theory for the reduction of animal testing

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Microphysiological systems (MPS), combined with control theory, offer an advanced framework for simulating pathological microvascular environments, particularly in chronic conditions like diabetes mellitus (DM). Traditional translational research often relies on animal models and static *in vitro* cultures, which fail to replicate the dynamic nature of vascular responses, leading to poor predictability in clinical applications. Computational modeling provides a mathematically rigorous approach to characterize microvascular hemodynamics, shear stress distribution, and inflammatory responses under diabetic conditions.

Control theory, typically applied in engineering, is now an essential tool for modeling the dynamic behavior of MPS, especially for microvascular systems. By integrating control theory into MPS, researchers can design systems that simulate biological processes and actively regulate conditions within the system to maintain physiological relevance. In diabetic microvascular dynamics, control theory helps simulate and predict how factors like blood flow, shear stress, and nutrient transport are altered in diabetes, providing insights into the mechanisms driving vascular complications.

This study presents an MPS-integrated computational approach for analyzing diabetic foot ulcer (DFU) microvascular dynamics using Navier-Stokes equations, non-Newtonian blood flow models (Carreau-Yasuda), and Laplace-transformed control system representations. The vascular microarchitecture was modeled in COMSOL Multiphysics, creating a parametric 3D vascular network with intrinsic roughness to capture localized shear stress gradients and flow instability zones. Pulsatile boundary conditions simulated cardiac cycle dynamics, revealing pressure differentials and vortex-induced particle dispersion linked to diabetic vascular impairment.

The CHIP-Eny platform, patented under BR 20 2025 004913 4, an Organ-on-a-Chip (OoC) system developed in collaboration with Universidade de Brasília (UnB) and Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), was used to validate computational predictions. This platform integrates endothelial cell cultures in a biomimetic microfluidic system.

The combination of MPS and control theory offers several advantages in predictive modeling. Control theory enables the integration of dynamic feedback loops, allowing for real-time adjustments to the simulated environment, thus improving the accuracy and reliability of models.

Moreover, combining control theory with MPS aids in developing predictive models for personalized treatments. Beyond preclinical



research, this approach holds potential for clinical use. By integrating predictive models into clinical decision-making systems, healthcare providers could use real-time data from patient monitoring to predict the progression of diabetic complications and adjust treatment plans accordingly. This enables a more dynamic and personalized approach to managing diabetes and its vascular complications, improving patient outcomes and reducing healthcare costs.

In conclusion, the integration of microphysiological systems and control theory for predictive modeling of diabetic microvascular dynamics offers a powerful framework for advancing our understanding of diabetes-related vascular complications.

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***In silico* tools for the assessment of steatotic chemical-mediated hepatocellular triglyceride accumulation**

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Introduction: Nonalcoholic hepatic steatosis, also known as nonalcoholic fatty liver disease (NAFLD), is one of the most common causes of chronic liver disease in the developed world and primarily affects men. Hepatic steatosis occurs when intrahepatic fat is $\geq 5\%$ of liver weight and is considered a consequence of the hepatic manifestation of metabolic syndrome and is associated with type 2 diabetes mellitus, obesity, and dyslipidemia. The main causes are diet, sedentary lifestyle, and chemical exposure to endocrine-disrupting chemicals (EDCs) such as

pesticides, plasticizers, metals, and perfluorinated compounds present in the environment. Up to now, animal models have significant limitations in reproducing the disease, so a computational model could significantly reduce the use of animals to determine the effects of various substances. From a molecular point of view, EDCs can trigger the xenobiotic activation of the pregnane-X-receptor (PXR) which is recognized as an adverse outcome pathway (AOP) for liver steatosis leading to hepatocellular triglyceride accumulation. Although there is strong evidence that PXR activation occurs primarily in the presence of mixtures of EDCs, some compounds can do so individually at concentrations as low as 3 mM. Accordingly, these EDCs should be of greater concern, and their early identification could be of great benefit in protecting global health.

Objectives: This work aims to apply international and European regulatory criteria for the assessment of bioaccumulation of chemicals based on octanol/water (logP) and octanol/air (logKoa) partition coefficients in the classification of steatotic xenobiotics that produce chemical-mediated hepatocellular triglyceride accumulation individually at low concentrations. For this purpose, machine learning tools will be developed for logP and logKoa prediction of eight EDCs of which two of them show pregnane-X receptor activation at 3 mM concentrations.

Materials and methods: A set of 14041 and 475 experimental values of logP and logKoa, respectively were compiled from previous works. All compounds were stored using their SMILES codes which were used to determine CDK and RDKit descriptors. Four feature selection methods were employed: Univariate Feature Selection (UFS), Recursive Feature Elimination (RFE), Sequential Feature Selection (SFS), and SHAP. Then, four different machine learning models were evaluated: Multiple Linear Regression (MLR), Random Forest (RF), Support Vector Machine (SVM), and XGBoost. Finally, the models were used to predict the logP and logKoa for eight EDCs (amiodarone, benzoic acid, cyproconazole, flusilazole, imazalil, prochloraz, propiconazole and tebuconazole).

Conclusions: Our machine learning metrics were able to rank the two steatotic xenobiotics that produced PXR activation at low concentrations (logP > 4 and logKoa > 10) out of the 8 total of EDCs.

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Development of human cardiac organoid-based preclinical platforms

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Human cardiac organoids (hCOs) derived from induced pluripotent stem cells (iPSCs) have emerged as powerful tools for preclinical research, offering the ability to mimic key aspects of human heart physiology and pathology in a controlled *in vitro* setting. In this study, we developed three-dimensional hCOs using a robust differentiation protocol and validated their potential as a platform for drug-induced cardiotoxicity testing. The organoids displayed contractile property and functional similarities to native cardiac tissue, including organized cardiomyocytes, endothelial cells, and fibroblasts, along with synchronized beating behavior and electrophysiological properties. We tested the applicability of hCOs as a cardiotoxicity model by assessing drug-induced toxicity, which can be classified into two major categories: drug-induced arrhythmias and cell death. The effects of drugs on ion channels within the cardiac membrane were analyzed, with an emphasis on identifying potential arrhythmogenic risks. Electrophysiological responses were further evaluated using a multi-electrode array (MEA) system, providing insights into extracellular field potential changes induced by the tested compounds. Given the critical role of mitochondrial ATP production in maintaining cardiac function, we also explored the effects of ATP depletion on organoid viability and function. Our results demonstrated that hCOs responded predictably to known cardiotoxic agents, validating their use in preclinical safety testing. The combined MEA and 3D viability assays confirmed that hCOs provide a reliable and scalable platform for evaluating cardiotoxicity in drug candidates, offering a promising alternative to traditional nonclinical models.

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Nanoencapsulation of antioxidants for ocular photoprotection: A cruelty-free approach using an *ex vivo* bovine cornea model

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Exposure to ultraviolet (UV) radiation threatens eye health, leading to diseases such as keratitis, cataracts and corneal degeneration due to oxidative stress, lipid peroxidation, protein degradation and cell death. Despite the scarcity of effective eye protection, more advanced strategies are needed to mitigate the damage caused by UV rays. L-Glutathione (L-GSH) and N-Acetylcysteine (NAC) have emerged as antioxidants capable of neutralizing reactive oxygen species (ROS) and reducing oxidative damage, but their limited stability and bioavailability hinder their effectiveness in topical applications. Nanoencapsulation in lipid systems has been explored as a promising method to increase the penetration and retention of these compounds in ocular tissues. Traditionally, the efficacy of ophthalmic formulations is evaluated in rabbits. In this study, we employed an *ex vivo* bovine cornea model to assess the protective effects of free and nanoencapsulated L-GSH and NAC, adhering to the 3Rs principle by eliminating the need for *in vivo* testing. Bovine corneas and lenses were extracted and exposed to UV-A radiation (17.2 W/m² for 3 hours at 32°C). Baseline opacity was measured, and the tissues were treated with varying concentrations of L-GSH (0.0625 mg/mL, 0.125 mg/mL and 0.25 mg/mL) and NAC (0.125 mg/mL, 0.25 mg/mL and 0.5 mg/mL), in both free and nanoencapsulated forms. After exposure, final opacity was assessed, and corneal permeability was analyzed by incubation with sodium fluorescein (4 mg/mL) for 90 minutes. The tissues were also subjected to morphological analysis (hematoxylin-eosin staining), mitochondrial activity (MitoTracker Red), ROS production and caspase-3 activation by indirect immunofluorescence, analyzed by High Content Analysis (HCA). The results showed that corneas exposed to UV-A for 3 hours exhibited significant increases in opacity and permeability, indicating substantial tissue damage. Treatment with L-GSH and NAC, at all concentrations, reduced these effects, with higher concentrations providing stronger protection. Nanoencapsulation improved photoprotection, resulting in reduced opacity, preserved epithelial structure and lower permeability. The combination of L-GSH and NAC exhibited a synergistic effect, providing superior photoprotection compared to the individual antioxidants. Histological analysis revealed better tissue preservation in the treated groups, especially with nanoencapsulated antioxidants. Immunofluorescence analysis showed increased mitochondrial activity, decreased ROS production and reduced caspase-3 activation in treated tissues, indicating less oxidative and apoptotic damage. Therefore, prolonged exposure to UV-A induced significant damage to the cornea, evidenced by increased opacity, permeability, oxidative stress and



cell apoptosis. Treatment with L-GSH and NAC, especially in nanoencapsulated forms, effectively protected the eye tissues. The combination of antioxidants showed a synergistic effect, increasing defense against UV-A damage. These findings highlight the potential of nanoencapsulation as an effective strategy for ocular protection against UV radiation, reinforcing the promise of nanoencapsulated antioxidants as a therapeutic approach to prevent UV-induced ocular damage. Moreover, this study underscores the importance of adopting cruelty-free methods in preclinical research to evaluate the efficacy of novel ophthalmic formulations.

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Development and characterization of an *in vitro* external retina co-culture model for the evaluation of systemic ocular toxicity

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Introduction: The eye, as an immunologically privileged organ, is protected by the blood-ocular barrier, which restricts the passage of systemically administered xenobiotics. However, in recent years, there has been a growing number of ocular complications associated with the use of systemic drugs. Although animal models, such as rodents and rabbits, are commonly used in these tests, they present ethical and physiological limitations, including the absence of specific structures, differences in the distribution and proportion of photoreceptors, vascularization, inflammatory and immune responses, as well as variations in drug metabolism. These differences highlight the need for more predictive and

effective models for assessing ocular toxicity in humans. In this context, *in vitro* models have gained prominence as a physiologically relevant alternative for evaluating the systemic effects of substances on the eye, helping to reduce reliance on animal testing.

Objective: To develop a co-culture model of external retina with ARPE-19 (a spontaneously immortalized human retinal pigment epithelium cell line) and HUVEC (Human Umbilical Vein Endothelial Cells) for the evaluation of systemic ocular toxicity.

Methodology: The co-culture model was set up in 8 mm transwell inserts, with ARPE-19 cells ($6 \times 10^4/\text{cm}^2$) on the apical side, cultured with a laminin coating, and HUVEC cells ($6 \times 10^4/\text{cm}^2$) on the basal side, cultured with a fibronectin coating. The transepithelial-transendothelial electrical resistance (TEER) was monitored for 21 days. Cellular viability was assessed using the LIVE/DEAD™ Viability/Cytotoxicity Kit. The model was characterized using ZO-1 (Zonula Occludens-1), Bestrofin-1, RPE 65 and CRALBP (Cellular Retinaldehyde-Binding Protein) antibodies for ARPE-19 and CD31 antibodies for HUVEC via immunofluorescence, and images were analyzed using a Leica SP8 confocal microscope.

Results: The co-culture model with ARPE-19 and HUVEC cells showed a TEER ranging from 24Ω to 115Ω over the 21-day culture period, with a stable peak of approximately 90Ω observed between days 4 and 14. The cellular viability assay showed that the cells maintained their viability throughout the experiment, with the majority of cells remaining viable over the 21 days. Characterization of the co-culture revealed proper cell organization and the expression of specific markers ZO-1 Bestrofin-1, RPE 65 and CRALBP (for ARPE-19) and CD31 (for HUVEC), confirming the functionality of the cells in the model.

Conclusion: Overall, our data demonstrates the effectiveness of the external retina model, presenting a functional barrier between ARPE-19 and HUVEC, preserving cellular viability and specific marker expression, thereby validating its application for the evaluation of systemic ocular toxicity. This model provides a relevant platform to test chronic exposure to potentially toxic substances, such as corticosteroids, offering a more accurate and ethical alternative to traditional animal models, thus improving predictive accuracy for human applications.

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Technological prospecting and systematic review of software using facial recognition with artificial intelligence in laboratory animals

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With advances in artificial intelligence (AI), new approaches to animal management, such as facial recognition software, are being explored. In humans, this technology is already applied in passport control, facial recognition on social networks, and smartphone unlocking. For laboratory rodents, some research groups have been using AI to develop assessment and welfare tools, either through Grimace Scales or by analyzing animal vocalization to identify signs of pain or distress. For non-human primates, these systems have been adapted for individual identification in both free-living and captive colonies, showing high success rates. In this context, the project “Development of AI-Based Technological Solutions for Assessing the Welfare of Animals Used in Biomedical Research” aims to create AI tools for facial recognition and welfare assessment of animals used in research. As a first step, technological prospecting and a review of scientific articles reporting AI-based computer vision software with a similar purpose to the project were conducted. Searches were carried out in patent and software databases (Google Patents and GitHub) and scientific production databases (Science Direct, PubMed, Google Scholar, and Europe PMC). Inclusion criteria considered only scientific articles and software records from 2014 to 2024 related to the keywords “facial recognition AND artificial intelligence AND animals.” Exclusion criteria included review and opinion articles, books, and any publications that were not research-based. The technological prospecting identified 16 software tools (12 records from Google Patents and 4 from GitHub), while the systematic review identified scientific article records as follows: Google Scholar = 2,500; Europe PMC = 891; Science Direct = 4; PubMed = 41. A total of 3,347 duplicate records were removed, along with 39 inaccessible articles and 50 AI-related articles on animals not used in research. After screening, 10 scientific articles describing the development of computer vision software for laboratory animal applications and 3 software records with the same purpose were identified. Despite the existence of some AI tools developed to refine animal experimentation, only one reported application for non-human primates in biomedical research. These results highlight that, although AI-based software for laboratory animals exists, only a small portion is specifically designed for non-human primates in research facilities, indicating a promising field for project development. The development of AI software for facial recognition and welfare assessment of research animals aligns with the ethical and technological demands of modern science, representing advances in the application of the 3Rs Principle.

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Reduction, replacement and refinement for regulatory purposes in relation to toxicity studies – an experience

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Even though a report was recently published in Argentinian national pharmacopoeia in relation with the application of alternative methods to the use of experimental animals for regulatory purposes, there are pharmaceutical products, not only drugs that require information about toxicity. For example, the manufacturer of some household products (products for cleaning, disinfection or to eradicate insects) must guarantee the safety of them using *in vivo* tests and specifically requires the demonstration that such products have a lethal dose 50 (dose que induce death in 50% of the treated animal) in rats greater than 2 g/kg for products in liquid form or 500 mg/kg in solid form.

Considering this situation, the objective of this work is to comply with current regulations, demonstrating the ability to obtain the required information reducing or even eliminating the use of laboratory animals. In this sense, different strategies are employed. First, a theoretical calculation of the required information is proposed based on safety data for the components and the percentage of each compound in the formulation, using mathematical formulas and data already reported in the literature to obtain the Lethal Dose 50 of the formulation.

Secondly, use *in vitro* cytotoxicity studies, particularly using the guide that allows estimate the initial dose to perform systemic toxicity studies (ENV/JM/MONO (2010) 20 if it is possible, due to potential interference that could invalidate the results obtained.

With all the information provided with the previous trials, it is possible to address the performance of an *in vivo* trial. In this sense, the next proposed step could be the use of different techniques that significantly reduce the number of animals, such as the up-down toxicity test (OECD guidelines 425), reducing the experimental subjects in a significant manner. At this step, it is possible to address the performance of a more complex *in vivo* trial (if the previous data were not enough to the health authorities) but only with the previously estimated dose and in order to confirm the safety results, adding, if it is necessary, not only the general response, but also clinical laboratory data of the experimental individuals, such as laboratory blood analysis (glycemia, uremia, creatinine, etc.) and histology of specific and relevant organs.

A similar strategy can be applied not only to household products but also to any other pharmaceutical product requiring safety information (chemical, household product, medical device, cosmetic, or medication).

In the case of drugs, although *in vivo* testing could be mandatory, the use of animals can be optimized, considering that the *in vivo* test could be designed with greater experimental evidence.

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COLAMA – Latin American Congress on alternative methods to animal experimentation: A reinforced experience in its fifth edition

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COLAMA has been a platform for over 20 years, bringing together the leading experts in alternatives to animal use and facilitating the dissemination of regional advancements. The event has been held in Brazil (1st edition in 2012), Cuba (2nd in 2016), Argentina (3rd in 2018), and Mexico (4th in 2022). In 2024, the organization of the event was assigned to Colombia, a country that has made significant regulatory and scientific progress regarding the improved use or replacement of experimental animals.

The event, primarily organized by the National University of Colombia, took place from September 16 to 20. It featured five pre-congress courses, attended by 162 participants, covering the following topics: 1) *In silico* methods as alternative tools to animal experimentation, 2) OECD GLP and validation of alternative methods, 3) New Approach Methodologies (NAMs), 4) Experimental design in animal research and 5) Alternatives to the use of animals in education and training.

Additionally, the main congress included five thematic blocks: 1) Bioethics, animal welfare, and refinement – the culture of care, 2) Alternatives to animal use in education and training, 3) Societal perspectives and perceptions regarding alternatives, 4) Replacement methodologies or New Approach Methodologies (NAMs) and 5) Alternatives in regulated industries, including a special session on NGRA, led by the ICCS.

The congress hosted 44 presentations and was attended by 164 participants from Argentina, Belgium, Brazil, Canada, Chile, Colombia, Costa Rica, Spain, the United States, France, Mexico, and the United Kingdom, who were affiliated with academia, associations, and the private or public sectors. Additionally, 39 scientific works were presented, either as oral presentations (12) or posters (27).

Additionally, a working session on regulation and the use of alternative methods was held, providing a space for dialogue and discussion on the opportunities, challenges, and difficulties in implementing animal replacement methods in research and education in Colombia. This session included the expertise of professionals from Brazil, Spain, and Argentina. One of the key outcomes was the proposal to create a network for alternative methods in Colombia, an initiative currently under development.

In total, the event featured 52 speakers, including 15 Colombians and 37 from other countries. A significant milestone was the signing of the Bogotá Declaration, calling on governments in the region to allocate greater attention and resources to promoting alternatives to animal experimentation. This declaration was signed by over 100 attendees.

COLAMA continues to strengthen its role as a key meeting space for the scientific community, government, industry, and society, fostering the exchange of experiences crucial for Latin America – a region that is actively engaging with and following global guidelines related to alternatives to animal experimentation.

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Sensitive detection of repeated dose hepatotoxicity using a simple on-chip perfusion liver MPS (BioStellar™ plate) with direct oxygenation

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In the field of drug development research, there is a significant demand for *in vitro* evaluation systems for drug-induced liver injury (DILI). However, a reliable model that accurately reflects physiological conditions has yet to be established. The main challenges include insufficient oxygen supply during cultivation and the absence of “flow” in the culture system, both of which are critical for maintaining hepatocyte functionality. Although polydimethylsiloxane (PDMS) has conventionally been used as an oxygen-permeable membrane, its high drug adsorption poses a major limitation, making it unsuitable for drug evaluation experiments where precise quantification is essential. These issues contribute to reduced sensitivity in assessing hepatotoxicity, hindering the development of safer drugs.

In this study, we aimed to develop a more reliable and simple liver MPS by combining micro-stirrer-based on-chip perfusion MPS (BioStellar™ Plate) [1,2] integrated with an oxygen-permeable InnoCell™ membrane to directly supply hepatocytes with sufficient oxygen. The InnoCell™ membrane is a new material that exhibits significantly lower drug adsorption [3], making it ideal for drug evaluation studies requiring minimal interference. Human hepatocytes, derived from humanized chimeric mice (PXB-cells), were cultured for two weeks in those conditions, during which various liver functions and responses to repeated doses of acetaminophen were evaluated to provide with a comprehensive assessment of hepatotoxicity.

PXB hepatocytes seeded on the BioStellar™ plates with InnoCell™ membrane demonstrated a significant increase in albumin secretion compared to the cells cultured on conventional oxygen-impermeable TCPS plates, enabling more sensitive toxicity detection in repeated doses of acetaminophen. This improvement highlights the enhanced physiological relevance of the new system. Additionally, we assessed the metabolic profile of acetaminophen, CYP activity, ALT activity in the culture medium, and the levels of glutathione and its oxidation products. The overall results indicate increased sensitivity in detecting acetaminophen-induced hepatotoxicity in the BioStellar™ plates with InnoCell™ membrane, showing a promise to improved drug safety screening.

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pH-responsive hybrid nanoparticles encapsulating *Stryphnodendron adstringens* extract: *In silico* 5-alpha-reductase inhibition and cytotoxicity assessment in BALB/3T3 fibroblasts

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Increased biological action of androgens in the skin can lead to dermatological disorders such as hirsutism, acne vulgaris, and androgenetic alopecia. These conditions can cause considerable psychological distress, significantly impacting self-esteem, mental health, and the social lives of affected individuals. Current therapies for these disorders present limitations in terms of efficacy and are often associated with undesirable side effects. One example is finasteride, considered the gold standard for treatment; although effective, it has teratogenic potential and can cause erectile dysfunction and other sexual function alterations. Thus, there is a clear need for new therapeutic strategies to manage these disorders. In this context, the objective of this work was to develop innovative products to treat these conditions, specifically pH-stimulus-responsive hybrid nanoparticles composed of silica (Si)/chitosan (Ch) and silica (Si)/sodium alginate (Alg), encapsulating the ethyl acetate fraction (EAF) obtained from the bark extract of *Stryphnodendron adstringens* (Mart.) Coville (Barbatimão), a plant native to the Brazilian Cerrado. In hair follicles, through the action of the 5-alpha-reductase enzyme, testosterone is converted into dihydrotestosterone (DHT), a functionally active and more potent form with a high affinity for androgen receptors. Therefore, inhibition of the 5-alpha-reductase enzyme represents an effective strategy for the treatment of these disorders. The Barbatimão extract is rich in catechins, which are promising inhibitors of the 5-alpha-reductase enzyme. Encapsulating EAF in nanoparticles appears to be a promising strategy to enhance follicular penetration and enable on-demand release at the pilosebaceous unit. Hybrid nanoparticles were obtained using Layer-by-Layer (LbL) method. EAF was characterized by HPLC/UV-Vis analysis. The inhibitory activity of the main compounds present in the EAF against the 5-alpha-reductase enzyme was investigated through *in silico* molecular docking studies. pH-responsive release from the particles were determined by UV-Vis spectroscopy. Cytotoxicity was assessed in terms of cell density/proliferation using neutral red and crystal violet assays, and cell morphology was analyzed by scanning electron microscopy (SEM). The levels of epigallocatechin-3-O-gallate (EGCG), epigallocatechin, and gallic acid in the EAF were found to be 32.0 ± 0.2 µg/mL, 30.0 ± 0.1 µg/mL, and 27.5 ± 0.2 µg/mL, respectively. EGCG demonstrated a higher binding affinity to the 5-alpha-reductase enzyme compared to finasteride, the gold standard treatment, suggesting greater inhibitory activity. The nanoparticles exhibited pH responsiveness, with EAF being more efficiently released from SiAlgEAF(LbL), sodium alginate-containing nanoparticle, at pH 7.4 – pH characteristic of the deeper region of hair follicle – releasing 61.9%, by the end of the study. Additionally, SiAlgEAF(LbL) were less cytotoxic compared to free EAF and the chitosan-containing particles, SiChEAF(LbL).

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Low-cost, non-animal-based molecular diagnostic tool for leprosy detection in resource-limited settings: A field-based alternative to footpad culture and PCR

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Introduction: Leprosy remains a neglected tropical disease (NTD) that continues to pose significant public health problems in South Asia. According to the Ministry of Health and Population of Nepal, more than 3,000 new leprosy case findings occur each year, yielding a prevalence of about 0.99 per 10,000 persons – above the World Health Organization elimination target in some of the provinces. This situation is compounded by Nepal's uncontrolled borders with India and China, whose total case counts are far higher and display a wide geographic spread. In particular, India accounts for over 50% of the leprosy burden in the world, whereas sporadic persistent cases continue to be common in the southern parts of China. Cross-border population dynamics and the lack of proper facilities of diagnosis in the Terai and Himalayan regions of Nepal pose significant obstacles to early and accurate diagnosis. Routine methods of diagnosis – such as polymerase chain reaction (PCR) or footpad culture involving mice or guinea pigs – are prohibitively costly, ethically problematic, and largely unavailable in rural health facilities.

The aim of this study is to evaluate a portable, cost-effective, and animal-free diagnostic technique that utilizes loop-mediated isothermal amplification (LAMP) to field-identify *Mycobacterium leprae* in endemic areas and in under-resourced settings in Nepal.

Methods: Retrospective study was conducted at a peripheral leprosy outreach centers. 21 leprosy suspects were recruited for the study. Skin smear and biopsy samples were subjected to a simplified DNA extraction protocol amenable to field use. LAMP amplification targeting *M. leprae*-specific sequences was performed using a portable heating block independent of electricity. The results were compared with conventional PCR methods and clinical diagnosis using WHO criteria. The study was done in strict adherence to the principle of the 3Rs, where laboratory animals were not used at any stage.

Results: The LAMP assay showed 91.4% sensitivity and 94.7% specificity compared to PCR methods. The colorimetric detection was simple, hence not requiring specialized laboratory technicians. The whole procedure was possible within 45 minutes and cost less than USD 2.50. In addition, the test did not require cold chain transport or laboratory equipment, successfully avoiding the use of animal experiments.

Conclusion: This study showed a field-deployable, non-animal-based diagnostic tool suitable for leprosy-endemic, resource-limited settings like Nepal. The LAMP platform offers a practical, ethical, and scalable solution to replace guinea pig footpad cultures and PCR for leprosy detection. This innovation is especially valuable in the Nepal-India-China tri-border region, where unregulated migration and shared disease burdens pose a critical challenge to leprosy control efforts. Implementing such tools can strengthen surveillance, promote early treatment, and support Nepal's national and regional elimination goals in alignment with the WHO NTD roadmap.

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Combined *in silico* and *in vitro* strategies for exploring an innovative organoselenium with anticancer potential

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In recent years, the development and application of alternative methods to animal experimentation have become relevant in scientific research, especially in the evaluation of safety and efficacy of new compounds. Notable approaches include *in silico* platforms, which use computational tools to predict biological properties, and *in vitro* methods, particularly those involving cell-based assays. These methods are promising alternatives, with the potential to reduce, refine, and replace the use of animals in research, aligning with the 3Rs principles. In this study, we conducted *in silico* and *in vitro* analyses for a new organoselenium compound, 5-Se-(phenyl)-3-(ferulic-amido)-thymidine (AFAT-Se). We analyzed its absorption, distribution, metabolism, excretion, and toxicity (ADMET), as well as the pharmacological and toxicological properties using different *in silico* platforms. Online platforms and tools, such as Molinspiration, pkCSM, admetSAR, SwissADME, WAY2Drug, CLC-Pred, DIGEP-Pred, STRING, Cytoscape and WebGestalt, were applied to predict biological activities. Multiple *in vitro* assays were conducted to verify the anticancer potential of AFAT-Se. Firstly, we conducted a monolayer/two-dimensional (2D) assay to evaluate the compound's cytotoxicity. HT-29 (human colon adenocarcinoma) and NCI/ADR-RES (human ovarian cancer; multi-drug resistant/MDR model) tumor cell lines were seeded in a 96-well plate, and treated with AFAT-Se for 72 h. For comparative purposes, the compound was encapsulated in poly(ϵ -caprolactone) (PCL) nanoparticles (AFAT-Se-NPs) and tested against the same cell lines, under the same conditions. MTT assay was performed, and the results were expressed as the percentage of cell viability. We also performed an *in vitro* wound healing assay, using NCI/ADR-RES cell line seeded in a 24-well plate. After 24 h, the monolayer was scratched with a pipette to resemble a wound. Then, the cells were treated with AFAT-Se-NPs and free AFAT-Se, and their migration rates were analyzed for 48 h. The *in silico* analysis evidenced that, according to Lipinski's rules, the compound exhibited great pharmacokinetic properties. It would be well absorbed by the intestine, it showed poor permeability in the blood-brain barrier, a high protein-binding rate, and it may interact with common drug metabolic enzymes, such as CYP3A4. AFAT-Se may also be a hERG inhibitor, with non-mutagenic, anticarcinogenic and chemoprotective properties. It could also strengthen the expression of the TP53 gene, and molecular targets associated with cancer mechanisms. The *in vitro* 2D assays corroborated that AFAT-Se has antitumor potential, particularly in the sensitive tumor cell line (HT-29) and at the highest tested concentrations. Notably, AFAT-Se-NPs dem-

onstrated enhanced antitumor activity, exhibiting significant cytotoxic effects against HT-29 cells even at lower concentrations, while also inducing a moderate reduction in the viability of MDR cells. Likewise, the wound healing assay showed that AFAT-Se-NPs were more effective than free organoselenium in inhibiting tumor cell migration, highlighting the potential effectiveness of NPs to suppress cell invasion in MDR tumors. Thus, compelling *in silico* and *in vitro* findings supported that AFAT-Se and, particularly, its nanotechnology-based drug delivery systems, emerge as promising anticancer strategies for circumventing MDR in cancer.

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New alternative method for predictive genotoxicity using a microphysiological system

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The assessment of genotoxicity for test compounds relies on a combination of tests designed to evaluate various endpoints associated with human diseases: mutagenicity, clastogenicity and aneuploidy. Currently, no single test can detect all mechanisms effectively, as existing assays involve rodent metabolic activation systems or *in vivo* rodent testing. This reliance can lead to discrepancies in human accuracy and predictive responses.

This study aimed to develop a single *in vitro* assay capable of accurately addressing both *in vitro* and *in vivo* genotoxicity endpoints using a human cells metabolically competent *in vitro* system to improve human-relevant predictivity and replace the reliance of animal testing. Different models were evaluated, and the most promising evaluated model consists of a fluidic flow microphysiological liver-on-chip system. This system, supported by CNBio's PhysioMimix™ platform, incorporated HepaRG cell line on specialized Liver Plates (MPS-LC12), which enhanced liver cells health and functionality compared to previous tested performed on Barrier Plate (MPS-T12).

Preliminary results using direct and pro-genotoxicants requiring metabolic activation demonstrated outcomes consistent with *in vivo* response. These included results from the comet assay, and micronucleus test. Key measurements, such as urea production and albumin secretion confirmed appropriate liver functionality and metabolic competence.

In conclusion, the human cells system using HepaRG cells in Liver Plates from CNBio represents a promising model, demonstrating appropriate metabolic properties without requiring additional metabolic activators. This system effectively addresses genotoxic adverse outcomes within a single system, i.e., induction of chromosomal damage or damage to the mitotic apparatus (micronucleus test) and DNA strand breakage (comet assay).

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Electro-metabolic coupling in multi-chambered vascularized human cardiac organoids

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The study of cardiac physiology is hindered by physiological differences between humans and small-animal models. Here we report the generation of multi-chambered self-paced vascularized human cardiac organoids formed under anisotropic stress and their applicability to the study of cardiac arrhythmia. Sensors embedded in the cardiac organoids enabled the simultaneous measurement of oxygen uptake, extracellular field potentials and cardiac contraction at resolutions higher than 10 Hz. This microphysiological system revealed 1 Hz cardiac respiratory cycles that are coupled to the electrical rather than the mechanical activity of cardiomyocytes. This electro-mitochondrial coupling was driven by mitochondrial calcium oscillations driving respiration cycles. Pharmaceutical or genetic inhibition of this coupling results in arrhythmogenic behavior. We show that the chemotherapeutic mitoxantrone induces arrhythmia through disruption of this pathway, a process that can be partially reversed by the co-administration of metformin. Our microphysiological cardiac systems may further facilitate the study of the mitochondrial dynamics of cardiac rhythms and advance our understanding of human cardiac physiology.

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A human-based skin-lymphoreticular model-on-chip to emulate inflammatory skin conditions

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Biomedical research is moving towards human-based disease models due to high drug development failure rates and limited translational value of traditional animal models [1]. Yet, despite fast-paced advances in bioengineering human-based models, emulating fine-tuned and inter-dependent immunoregulations of the human body remains challenging. While many bioengineered models contain immune cells, they often rely on a limited set of predefined cell types and lack interactions with secondary lymphoid tissues.

Here, we report a self-assembled, minimally functional lymphoreticular unit composed of primary human fibroblastic reticular, lymphatic endothelial and CD4+ T cells that emulate structural and functional interactions with the skin under inflammatory conditions. Following the optimization of the culture conditions, a layer-by-layer approach combining the relevant cell types yielded a self-organized and compartmentalized lymphoreticular model mirroring human lymphoid tissue. Following verification of T cell motility, we established a skin-lymphoreticular co-culture within a microfluidic organ-on-chip to mimic interactions between skin and its draining lymph nodes. Co-cultivation with atopic-like skin models and the topical application of the skin sensitizer 2,4-dinitrochlorobenzene stimulated strong migration and T cell infiltration from the lymphoreticular models while maintaining tissue integrity. The addition of anti-inflammatory drugs abrogated the T cell mediated effects verifying the functionality of the model system. Taken together, the presented human-based skin-lymphoreticular model resembles a minimally functional unit that allows to study interactions between the skin and adjacent lymphoid tissues in a human-based setup.

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Presentation: Poster



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Biological safety and anticancer potential of nanoformulations containing an innovative organoselenium: *In silico* and *in vitro* studies

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The need for new antineoplastic therapies has been increasingly discussed, as cancer remains a global health concern. Innovative molecules have been sought to address issues such as low selectivity to tumor tissue and unwanted side effects. Alternative methods then emerge as a way to screen and evaluate the activity, safety, and specificity of these molecules. This study aims to encapsulate the innovative organoselenium 5'-Seleno-(phenyl)-(3-N)-5'-Seleno-(4-methyl-phenyl)-4-N(6-chloro-1,3,5-triazin-2-yl)-thymidine (Di3a) in a polymeric nanostructured system (NC-Di3a) and in a lipid system (CLN-Di3a), and to perform a comparative evaluation of the antitumor potential and biological safety of these formulations using *in silico* and *in vitro* approaches. The Molinspiration, pkCSM, admetSAR, and SwissADME databases were applied for *in silico* prediction of the pharmacokinetic properties of Di3a. We employed the MTT assay to evaluate cell viability of tumor and non-tumor cell lines (A549, lung cancer; NCI/ADR-RES, ovarian adenocarcinoma; L929, murine fibroblasts) after incubation with the formulations. The hemolysis assay assessed the biocompatibility of the NCs and CLNs and served as an endosomal membrane model to verify the pH-dependent behavior of the formulations containing the surfactant 77KL, noting changes in cell membrane rupture. Concerning the Lipinski rule of five, the organoselenium violated some parameters, such as molecular weight exceeding 500 g/mol, log P > 5, and HBD > 10. The main pharmacokinetic characteristics observed included low absorption in the gastrointestinal tract, potential substrate of P-gp, poor distribution across the blood-brain barrier, probable substrate and inhibitor of CYP3A4, and a possible hERG II inhibitor. In the *in vitro* studies, the formulations exhibited concentration-dependent antiproliferative activity against A549 cell line. CLN-Di3a demonstrated greater cytotoxicity, with ~12% cell viability at the concentration of 100 µg/mL, while NC-Di3a exhibited ~23%. In the resistant/MDR NCI/ADR-RES cell line, both formulations showed moderate cytotoxicity, with cell viability of ~80% and ~82% for NC-Di3a and CLN-Di3a, respectively, at the highest concentration. Furthermore, the formulations did not display a cytotoxic effect on the non-tumor L929 cell line, showing cell viability > 100% at all tested concentrations, which highlights the tumor-selectivity of the nanoformulations. The nanocarriers also exhibited biocompatibility in the hemolysis assay, with hemolysis rate lower than 5% at a concentration of 250 µg/mL. NC-Di3a and CLN-Di3a demonstrated higher hemolytic potential at pH 5.4 than at physiological pH, evidencing, thus, their ability to lysis the endosomal membranes for intracellular drug release. None of the formulations lacking the surfactant 77KL demonstrated hemolytic activity, irrespective of the concentration and pH evaluated. Thus, the *in vitro* assays evidenced

the biocompatibility of the nanoformulations, while both *in silico* and *in vitro* studies provided valuable insights into the potential of the NCs and CLNs containing the innovative molecule Di3a as antitumor strategies to overcome current problems of conventional cancer therapies.

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In vitro comparative study of the role of polymer type and surfactant composition on the toxicological profile of nanoparticles

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Nanoparticulate systems have unique properties, which makes them interesting for diverse biological applications. When used in biomedical contexts, these systems constantly interact with biological surfaces. Such interactions can enhance the delivery of loaded drugs but may also lead to significant toxic effects. Many studies have been describing data on the toxicity pattern of nanoparticles (NPs); however, the great majority described the safety evaluations for drug-loaded NPs, without information for unloaded counterparts. Therefore, it is of utmost importance to comprehend the toxicological effects of a blank NP on cell membranes before its proposal as a potential carrier for drug delivery. In this research, we evaluated NPs formulated as nanospheres and nanocapsules and investigated how the composition of polymers and surfactants affects their toxicological profile, using *in vitro* new approach methodologies (NAMs) to study cytotoxicity and biological safety. We employed the hemolysis assay to evaluate the formulation's hemocompatibility and biocompatibility, while the MTT assay was used to assess the NPs cytotoxicity in a 3T3 cell line (murine Swiss albino fibroblast). Our results showed that the type of polymer used to prepare the NPs significantly impacts their properties, especially regarding cell tolerance. In general, NPs based on Eudragit® RL 100, a cationic polymer, exhibited greater cytotoxicity and hemolysis rates than those based on PCL, PLGA, and chitosan. This highlights the critical role of polymer selection in determining toxicity. Furthermore, including the surfactant Span 80® in the NP matrix increased its toxic effects, which also emphasizes the importance of surfactant choice in NP design. Both nanospheres and nanocapsules based on the same polymer displayed comparable toxicological profiles. Also, *in vitro* hemolytic and cell cytotoxicity assays, used to study the biological security of the NPs, showed convergent results, which could demonstrate a correlation between hemolysis and toxicity. Therefore, our data suggest that NP constituents can critically contribute to its toxicity, highlighting the importance of *in vitro* toxicological and safety studies to better understand the effects of nanoformulations.

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Challenges and opportunities at the intersection of artificial intelligence (AI) and alternative methods to animal use: A past, present, and future perspective

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Introduction: The intersection of artificial intelligence (AI) and alternative methods to animal use, including New Approach Methodologies (NAMs), represents a transformative frontier in scientific research. Historically, animal models have been indispensable for understanding biological processes, disease mechanisms, and drug development. However, ethical concerns, regulatory pressures, and scientific limitations have driven the search for alternatives. The rise of AI has opened new possibilities for reducing and replacing animal use, offering innovative tools for predictive modeling, data analysis, and simulation.

Objective: This work aims to explore AI's transformative potential in advancing alternative methods, presenting a critical analysis of past breakthroughs, current innovations, and future directions.

Methods: A narrative, three-phase temporal analysis (past, present, future) of peer-reviewed literature, conference proceedings, and white papers (2010-2024) was conducted using data from PubMed, IEEE Xplore, and OECD reports.

Results: In the past, early efforts to replace animal use focused on *in vitro* models (e.g., cell cultures) and computational methods like QSAR modeling. While groundbreaking, these approaches struggled to replicate complex biological systems. The advent of AI in the late 20th century improved predictive capabilities but was limited by computational power and data scarcity. Today, AI is revolutionizing alternatives through machine learning (ML), deep learning (DL), and big data analytics. Key advancements include AI-enhanced organ-on-a-chip and 3D bioprinting for human physiology simulation, AI-driven toxicology and drug screening (e.g., AlphaFold for molecular interaction prediction), and omics data integration for comprehensive biological modeling. Looking ahead, AI's role in alternatives is promising but requires addressing critical challenges: data standardization for reliable AI training, explainable AI (XAI) for regulatory trust, multidisciplinary collaboration between AI and life sciences, and ethical governance of synthetic data. Emerging technologies like quantum computing and advanced neural networks could further enhance AI's predictive power.

Conclusion: The convergence of AI and alternative methods offers a triple-win scenario: superior human-relevance, enhanced ethics, and improved accuracy. Future success hinges on overcoming technical hurdles (e.g., rare endpoint data), ensuring model interpretability, establishing validation frameworks, and fostering global collaboration. By addressing these challenges, AI-powered, human-centric approaches can become the new gold standard, achieving both scientific excellence and ethical responsibility.

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In vitro assessment of DILI using human-relevant HepaSH spheroids derived from humanized liver chimeric mice

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Background: Drug-induced liver injury (DILI) is a major cause of post-marketing drug withdrawal, despite nonclinical DILI risk assessments primarily conducted in animals, thereby indicating the need for development of human-relevant *in vitro* models. Primary human hepatocytes (PHHs) and 3D cultures, like spheroids, have been reported to be useful for DILI prediction; however, lot-to-lot variations and inconsistent availability of PHHs are significant limitations. In contrast, HepaSH cells, human hepatocytes derived from humanized liver chimeric mice, are more consistently available and exhibit gene expression profiles of drug-metabolizing factors similar to PHHs with minimal lot variation. Thus, HepaSH cells may be promising alternative tools for assessing DILI risk.

Purposes: We aim to uncover whether HepaSH cell-derived spheroids can be used to assess DILI risk in humans.

Methods and results: HepaSH cells were cultured in two-dimensional (2D) and three-dimensional (3D) conditions for up to 21 days. Gene expression analysis revealed that HepaSH spheroids exhibited enhanced mRNA levels of drug-metabolizing enzymes, albumin, and transporters. Correspondingly, the enzymatic activity of CYP3A4, a major cytochrome P450 enzyme, and albumin secretion were both higher in 3D-cultured HepaSH cells compared to 2D-cultured cells. Cytotoxicity was assessed by measuring intracellular ATP levels following exposure to DILI-associated drugs from the DILIrank public dataset. HepaSH spheroids cultured in a chemical-defined medium exhibited the highest sensitivity to drugs classified as having a high risk of DILI.

Conclusions: Our data demonstrate that hepatocytic spheroids generated from HepaSH cells are suitable for *in vitro* DILI risk assessment. A large-scale compound screening study would be required to clarify the predictability of HepaSH spheroids as a non-clinical testing method for assessing human DILI risk.

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Organ-on-chip and natural products: A sustainable and ethical frontier for drug discovery

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Organ-on-chip (OoC) technology is emerging as a powerful tool in health research, particularly for evaluating natural products. This innovative approach, which bridges biotechnology and herbal pharmacology by simulating human physiology on microfabricated platforms to enable precise studies of substance interactions within the body, aligns with the One Health approach, recognizing the interconnectedness of human, animal, and environmental health and their shared influence on overall well-being. OoCs combined with research on natural products holds great promise in efficacy and safety evaluations for bioactive compounds obtained from plant extracts. Proving scientifically these activities becomes very difficult due to its complexity when dealing with biological systems. Recent reviews have described such organs being used for efficacy evaluation, toxicity evaluation, and metabolism evaluation concerning natural products. The most important part of this plan is how synthetic biology and AI join forces with OoCs to not only create new compounds but also to improve existing formulations, thereby maximizing the inherent therapeutic power of natural plants. Despite advancements in OoC technology, challenges remain regarding standardization, validation, and scalability, alongside biases favoring traditional methods. In essence, OoC technology accelerates the discovery of new natural product-based treatments while reducing reliance on animal testing, enhancing our understanding of biocompatibility, and elucidating the interactions within complex plant extracts. Overall, OoC technology provides a foundational platform for the development of diverse bioactive natural products and introduces a new paradigm for the assessment of next-generation natural drugs. Integrating OoC with natural product research paves the way for more effective and safer treatments, contributing to an overall improvement in quality of life through comprehensive healthcare solutions. Brazil's rich biological diversity is often cited as a key competitive advantage because it offers an almost inexhaustible source of bioactive compounds with significant therapeutic potential. Achieving this potential requires enhanced cross-sector collaboration among research institutes, pharmaceutical industry and regulatory agencies. Such multidisciplinary partnerships could develop enhanced health products and therapeutics from native medicinal plants contributing to public health. To fully realize this potential, adopting innovative strategies such as integrating digital technologies, the principles of One Health, and 3R is essential. These approaches are key to Brazil exploring its potential with natural products, fostering sustainable and innovative healthcare solutions rooted in its rich and diverse natural heritage.

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In vitro evaluation of the genotoxicity and cellular effects of topical formulations with red propolis

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The search for alternative treatments for wound healing, particularly in burn injuries, has led to a growing interest in natural products due to their biocompatibility and therapeutic potential. In this context, Olivem[®]1000, a commercial emulsifier derived from olive oil, and red propolis extract from Alagoas, Brazil (SISGEN no. A305815), have emerged as promising candidates for topical application. The aim of this study was to evaluate the *in vitro* toxicity and cellular effects of two formulations: the OL (Olivem1000) and OLRP (Olivem 1000 combined with red propolis extract) were utilized in a study involving adult mouse fibroblast cells (L929 cell line). The cells were cultivated in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, maintained at 37°C in a 5% CO₂ atmosphere. The conditioned media were prepared through serial dilutions of the formulations and applied to the cells for 24 hours. The assessment of cell viability was assessed using the MTT assay, in accordance with the ISO 10993-5:2009 guidelines, which enabled the determination of the IC₅₀ (40 µg/mL). To further investigate cellular responses, genotoxicity, cytoskeletal organization, and cell migration assays were performed. Cells that had been exposed to conditioned media at the IC₅₀ concentration were incubated for 24 h. Thereafter, they were stained with Hoechst 33342 and Actin-Green 488 following which they were analyzed via confocal microscopy. The quantification fluorescence was conducted using ImageJ software. No genotoxic effects were observed ($p > 0.05$), and nuclei remained intact and morphologically preserved. A greater number of mitotic cells were observed in both the OL and OLRP groups compared to the control, evidenced by nuclear condensation and actin reorganization, features associated with cell proliferation. The migration of cells was evaluated using the scratch wound assay. A linear scratch was created in confluent monolayers. Thereafter, the cells were treated with conditioned media and monitored over a 24 h period. Image analysis revealed that the treated groups exhibited accelerated wound closure, with complete closure observed at 72 h. In contrast, the control group exhibited a slower closure rate, with the wound remaining open up to 90 h. In conclusion, both formulations demonstrated biocompatibility and an absence of cytotoxic or genotoxic effects. However, the formulation containing red propolis (OLRP) showed enhanced stimulation of fibroblast proliferation and migration, suggesting that the presence of this bioactive compound potentiates the cellular response. These findings highlight the potential of red propolis as an adjuvant in regenerative dermatological formulations.

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Enhancing a curated high-throughput screening pipeline to improve toxicological interpretations of *in vitro* data

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Testing of thousands of chemicals in the ToxCast and Tox21 high-throughput screening (HTS) programs has generated millions of data points that can be difficult to analyze in detail. To increase confidence in HTS bioactivity calls, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) has reviewed these data to generate a curated HTS (cHTS) data set, in which results are flagged based on chemical quality, curve fit, or potential technological interference. In this presentation, we will describe recent improvements to our curation process that have been made to accommodate major updates in concentration-response modeling in the U.S. Environment Protection Agency (EPA) ToxCast/Tox21 database (Feshuk et al., 2022).

All data were retrieved from the EPA invitrodb v4.2 database and analyzed using the *tepl* R package. Representative curves were derived for each chemical-assay pair, and assays that were determined to have limited biological relevance were removed. Removals included, but were not limited to, background readouts used for assay quality control; individual channel readouts (only ratios were retained); and transcriptional activation assays analyzed inappropriately in the loss direction.

Remaining assays received “Flag-Omit” labels if assay performance or curve fit was deemed not biologically relevant. Examples included estimated points-of-departure that exceeded the tested range, mismatches between curve direction and the intended direction of analysis, or down-direction curves where the best-fit model was gain-loss. We also obtained quality control (QC) data on chemicals tested and applied them to the curation process. Curves were flagged as “QC-Omit” if the sample or chemical was graded as low concentration or low purity during the QC process. Finally, chemical-assay pairs were examined for potential technological interference, as luciferase inhibition and fluorescence quenching/autofluorescence can result in false bioactivity signals. Assays using these technologies were identified via assay metadata and literature review. Potential interferent chemicals were identified via Tox21 tests or predicted using quantitative structure-activity relationship models (Borrel et al., 2020). If a chemical was interferent for a technology and was tested in an assay using that technology, the assay-chemical pair was flagged with “Technological Interference.” This curation results in at least one flag for 46% of the 1,865,056 assay-chemical pairs.

We also annotated cHTS data to mechanistic targets that facilitate linkage to modes-of-action and subsequently to toxicological outcomes of regulatory interest. All mechanistic target and mode-of-action terms were mapped using the Open Biological and Biomedical Ontology Foundry. We considered curve direction in this annotation, as an increased or decreased response can change the biological interpretation.

This rigorous curation process ensures that bioactivity calls are robust, interpretable, and biologically relevant. Updated cHTS data are available in NICEATM’s Integrated Chemical Environment (ICE, <https://ice.ntp.niehs.nih.gov/>), an open-access resource for toxicologically relevant data and computational tools.

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A cytotoxicity assay as an alternative to the murine model for the potency testing of *Bothrops jararaca* venom and antivenom: An intralaboratory pre-validation study

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Antivenom therapy is the only specific treatment for snakebite envenomation, and antivenom potency determination is key in the efficacy assurance quality control process. Nowadays, this process relies on the *in vivo* murine model – thus, the development of alternative *in vitro* methods is imperative. In the current study, the principle of the proposed method is the ability of *Bothrops* venom to induce cytotoxic effects in Vero cells, and the capacity to evaluate the inhibition of this cytotoxicity by the respective antivenom. After exposure to the venom/antivenom, the relative proportions of adherent (viable) cells were evaluated by direct staining with Coomassie Blue. The optical density (OD) of the lysed cell eluate was directly proportional to the number of adherent cells. This cytotoxicity-based alternative method could represent a potential candidate for validation as a replacement for the current *in vivo* test. The *in vitro*-determined cytotoxicity of the Brazilian *Bothrops* reference venom (expressed as the 50% effective concentration; EC50) was 3.61 µg/mL; the *in vitro*-determined 50% inhibitory concentration (IC50) of the Brazilian *Bothrops* reference antivenom was 0.133 µL/mL. From these two values, it was possible to calculate the potency of the reference antivenom. The results from the assays exhibited a good linear response, indicating that the method could be a potential candidate replacement method for use in antivenom quality control prior to lot release, subject to further validation.

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Innovation in vaccine potency assessment: Comparison of regulatory frameworks and application of animal-free methods

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Vaccination remains the most effective tool for preventing infectious diseases. Potency testing is a key step in ensuring vaccine efficacy, traditionally carried out using *in vivo* challenge models. While these methods are established in pharmacopoeias and WHO guidelines, they require large numbers of animals and pose concerns related to scientific relevance, reproducibility, and ethical acceptability. This study compares the use of *in vivo* potency assays and the availability of validated alternatives in the Brazilian Pharmacopoeia (BP), the European Pharmacopoeia (EP), and WHO technical documents. We reviewed these sources to identify vaccines for which *in vivo* testing is still prescribed and assessed the extent to which animal-free or animal-reducing approaches have been adopted. Although *in vivo* methods are still described across all compendia, EP and WHO now recommend alternative approaches, including ELISA-based antigen quantification, single- or multiple-dilution serological assays, and *in vitro* cell-based systems. These alternatives have shown potential to reduce animal use by over 70% in some vaccines. In contrast, the BP continues to rely heavily on animal-based protocols, even when internationally validated methods have proven to be equivalent or superior in performance. Our findings reveal a substantial lag in Brazil's implementation of the 3Rs (Replacement, Reduction, and Refinement) in vaccine potency testing. Even for products where *in vivo* methods are still in global use, Brazil has yet to adopt animal-reducing strategies like single-dilution protocols or validated *in vitro* assays. Given the flexibility provided by ANVISA's RDC 511/2021, which allows for the use of internationally recognized compendia, Brazil has the opportunity to incorporate alternative methods with partial validation, without compromising vaccine quality. This study underscores the urgent need for regulatory alignment and enhanced collaboration between pharmacopoeial bodies, regulatory authorities, and manufacturers to overcome scientific and operational barriers and promote the broader adoption of humane, modern testing strategies.

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Developing and designing INVITES-IN; a tool for assessing the internal validity of *in vitro* studies

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Background: Applying systematic review principles in chemical risk assessment has become an established methodology for achieving rigorous, transparent and objective analysis of all evidence relating to the assessment task. An important part of the review methodology is the evaluation of internal validity, i.e., the extent to which the design, conduct, and reporting of a study are likely to have prevented bias. A tool for the evaluation of internal validity of *in vitro* studies is needed.

Objective: To systematically and transparently design and develop a tool for the evaluation of the internal validity of *in vitro* studies. The tool is called INVITES-IN (*In vitro* Experimental Studies Internal validity).

Methods: The conceptualization and development method includes four sequential studies, involving domain experts and potential end-users of the tool:

- Study 1. Creating a comprehensive database of issues that may affect the internal validity of *in vitro* studies of any design.
- Study 2. Prioritizing issues from the database according to their importance to the assessment of internal validity of *in vitro* studies.
- Study 3. Creating a beta version of an appraisal tool based on analysis of the prioritized issues identified in stage 2.
- Study 4. Creating a final release version of the tool, based on user-testing of the beta version.

Results: In total, 405 issues of relevance for introduction of bias to *in vitro* studies were identified and included in an item bank (study 1). Of these, 372 were considered to be of importance for the introduction of bias to *in vitro* studies (study 2). The creation of the beta version of INVITES-IN is currently ongoing.

Conclusions: The importance of involving domain experts in tool development is demonstrated from the two completed studies (study 1 and study 2). The methodology applied for the creation of INVITES-IN is considered to be useful for development of appraisal tool development in general.

INVITES-IN will provide a structured approach for retrospective study validity assessment. It will i) support objectivity, transparency and openness in the process of summarizing evidence for decision making, ii) support the incorporation of evidence from *in vitro* data in chemical risk assessments and pave the way for the use of such data as stand-alone evidence (both guideline and non-guideline studies), iii) contribute to confidence in the use of NAMs data as evidence in chemical risk assessments.

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Identification of anti-TB therapy induced ADRs genetic markers using *in silico* approaches

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Introduction: Adverse drug reactions (ADRs) are associated with clinical morbidity and, in severe cases, even mortality. Globally billions of dollars are spent on managing these ADRs for common and uncommon diseases. Due to these reasons drug resistant strains have emerged and are now a serious challenge to TB eradication. To effectively deliver the available treatment regimen and ensure patient compliance it is important to manage ADRs more efficiently. Recent studies have demonstrated that drug outcomes are patient-specific and can, therefore, be predicted. A few of these drugs, including a few administered for TB, have shown excellent correlation with response rates and development of ADRs.

Method: ADRs selected based on frequency of occurrence ($\geq 1\%$). Anti-TB drugs were reviewed to identify the candidate genes (DMETs, HLA). Genes analyzed with different web tools and databases to extract their SNPs. MAF > 0.01 shortlisted using NCBI Gene and dbSNP databases (built 141). SNPs which lay in a functional domain of the gene were prioritized using SNPinfo web server (www.snpinfo.niehs.nih.gov/). Additionally, same analysis was done for Indian population.

Result: We identified 10 genes which maybe directly linked to ADRs to various anti-TB drugs, 4 of these have been documented earlier. Nearly 47 genes were identified for indirect association with ADRs by virtue of them being off-targets of the drugs. Lastly, 5 genes were reported for their allelic association with anti-TB DIH. To our knowledge, this is the first review reporting a list of possible genetic markers in context to TB ADRs to such a vast extent.

Conclusions: New genes are identified that may be associated potentially with anti-TB drug ADRs.

This would translate into not just patient welfare but would also save billions of dollars spent annually on managing drug induced ADRs.

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Employability of alternative methods for replacing animal tests in the regulatory evaluation of veterinary products in Brazil

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Veterinary products are defined as any chemical, biological, or biotechnological substances, as well as manufactured formulations, intended for individual or collective administration – either directly or through feed – for the prevention, diagnosis, treatment, or cure of animal diseases. This category also includes zootechnical additives, performance-enhancing supplements, pharmaceuticals, vaccines, antiseptics, disinfectants, pesticides, and substances for cosmetic or physiological modulation in animals. In Brazil, veterinary products are regulated by the Ministry of Agriculture, Livestock and Food Supply (MAPA), which establishes technical and regulatory standards for their production, quality control, commercialization, and use. The registration of such products requires a range of safety and efficacy tests, often including *in vitro* and *in vivo* studies on non-target species. A notable example is the innocuity test in guinea pigs, frequently mandated for veterinary immunobiologicals. Brazilian legislation concerning the ethical oversight of animal use in veterinary testing remains outdated and disconnected from evolving global standards. In contrast, national and international regulations related to human products have already incorporated validated non-animal alternative methods across both development and quality control phases. Considering the increasing societal and scientific concern for animal welfare and the growing perception of animals as family members, there is a pressing need to identify regulatory assays that can be replaced by scientifically validated non-animal approaches. This initiative aims to support the development of a technical proposal to be submitted to regulatory authorities, promoting the revision of current regulations and the progressive incorporation of scientifically validated alternative methods into the national regulatory framework as replacements for traditional animal-based testing. A narrative literature review will be carried out, guided by selected elements of systematic review methodologies, to critically and comprehensively synthesize the available scientific evidence related to alternative methods for veterinary product testing. The expected outcome is a regulatory-oriented proposal demonstrating the feasibility and applicability of alternative methods for replacing animal tests in the registration of veterinary products, particularly for safety and quality assessments currently mandated by Brazilian legislation.

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The TXG-MAPr toolbox: Qualitative and quantitative interpretation of high throughput transcriptomics for NGRA hazard assessment

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Chemical-mediated adversities are generally associated with cellular perturbations that lead to specific cell (stress) signaling pathways that drive diverse transcriptional programs. Therefore, transcriptomics is an ideal technology to define compound specific mode-of-action for chemical hazard assessment. Interpretation of transcriptomics is typically performed using genuine pathway enrichment tools, thereby excluding the presence of cell type specific transcriptional regulatory networks. As a consequence, transcriptomics interpretation for hazard assessment would require approaches that take the cell- and tissue-specific responses into account. We have established a TXG-MAPr interpretation toolbox for HTTr interpretation. The TXG-MAPr tool is based on weighted gene co-regulation network analysis (WGCNA) of large HTTr datasets from diverse chemical treatment for individual human *in vitro* test systems. We have established a TXG-MAPr tool for different liver (HepG2, iPSC-hepatocyte like cells, primary human hepatocytes), kidney (RPT-EC-Tert1 and iPSC-proximal tubule like cells) and neuronal (LUHMES cells) and immune cells (fresh peripheral blood mononuclear cells (PB-MC)). The tool provides comparison with existing HTTr data as well as compound comparison within a new substance class. In different case studies we demonstrate the TXG-MAPr tools facilitate the qualitative and quantitative interpretation of complex transcriptomics set and allow the application in diverse regulatory relevant hazard approaches including prioritization of highly toxic compounds and read across and grouping. The TXG-MAPr toolbox allows HTTr data interpretation by non-bioinformaticians including chemists, biologists and regulators. Future work will focus on expanding the TXG-MAPr tools a broad range of toxicity testing relevant test systems.

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Endogenously tagged human stem cell reporters for high-content screening of cellular stress responses for next generation target organ toxicity safety assessment

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Predicting systemic target organ-specific toxicities (STOT) is a critical challenge in chemical safety assessment. In this study, we aimed to develop a novel *in vitro* test battery capable of identifying different cellular stress responses and elucidating the mechanisms of action of chemical stressors. By utilizing human induced pluripotent stem cell (hiPSC)-derived organ-specific cell types – hepatocyte-like cells (HLCs), cardiomyocytes, and renal proximal tubule-like cells (PTLCs) – we focused on profiling four different cellular stress-response pathways essential in toxicity to aid in early-stage hazard identification. We performed comprehensive transcriptomic profiling of hiPSC-derived HLCs, cardiomyocytes, and PTLCs following exposure to chemical stressors of adaptive stress response pathways. Differential gene expression analysis enabled us to identify key stress-responsive biomarker genes activated across all three lineages, forming the basis for developing a targeted hiPSCs stress reporter panel, designed to capture stress pathway activation through real-time monitoring. Utilizing CRISPR/Cas9 technology, we generated a fluorescent reporter system incorporating these biomarker genes, specifically linked to pathways involved in inflammatory signaling, DNA damage response, endoplasmic reticulum (ER) stress, and oxidative stress response, each tagged with enhanced green fluorescent protein (eGFP). After differentiation into organ-specific cell types, the reporter panel enabled real-time tracking of pathway activation in response to chemical exposures, using live cell confocal imaging. We observed distinct dose-dependent responses with biomarker-eGFP-positive cells appearing in each lineage. We further explored the application of these reporters in iPSC-derived 3D organoids, including kidney and mammary gland organoids. We exemplify the application of these reporters in both liver and kidney specific toxicity responses in both 2D and 3D models. This rigorously characterized hiPSCs reporter platform provides an *in vitro* assay that can capture organ-specific responses to toxicants, providing mechanistic insight into the cellular pathways activated upon compound exposure.

This work received funding from the European Union's Horizon 2020 project RISK-HUNT3R (Grant Agreement No. 964537), which is part of the ASPIS cluster, and the Horizon Europe project PARC (Grant Agreement No. 101057014), and the Horizon Europe 2021 – Cancer Mission DISCERN project (Grant Agreement No. 101096888).

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Sanctuaries: A dignified end of life for animals after experimentation

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Often the animals that have undergone experimentation are healthy, especially those that were used as controls. Directive 2010/63/EU states that “at the end of the procedure, the most appropriate decision must be taken as to the future of the animal on the basis of its welfare and potential risks to the environment”. It also mentions that animals that have their well-being compromised should be euthanized. The same Directive also mentions that animals that are healthy must be sent to their habitats, an appropriate zootechnical system and, in the case of dogs and cats, they must be sent for adoption. In Brazil, Law 11,794/2008 recommends that animals be euthanized at the end of the experiment, however, it makes it possible to deliver the animals to animal protection societies or reputable people. We must consider that many animals have a very high cost such as horses, non-human primates, sheep and goats. A horse that has been used for the production of antibodies to hyperimmune sera can be used, for example, in hippotherapy. Other large animals can go to small farms where children can be put in contact with nature and environmental education. In view of the possibilities offered by European and Brazilian legislation and knowing that not all protective societies are able to receive these animals, the creation of sanctuaries or the reception of animals by existing sanctuaries is of paramount importance to give a dignified end to the large laboratory animals.

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NAMs for anticancer drug evaluation: A predictive platform for veterinary precision medicine

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Spontaneous tumors in companion animals, particularly dogs, represent valuable models for translational oncology. In veterinary oncology, especially in cases of malignant melanoma, a highly aggressive and treatment-resistant neoplasm, there is a critical need for platforms that can evaluate chemotherapeutic efficacy in a patient-specific context. This study aimed to establish and validate primary 2D and 3D *in vitro* models derived from canine melanoma tissue to assess the cytotoxicity of vincristine and doxorubicin, supporting future implementation of personalized veterinary oncology strategies. Primary cultures were generated from surgically excised fresh melanoma tissue by mechanical and enzymatic dissociation using trypsin. The resulting cell suspension was seeded in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics. For the 2D model, cells were cultured in standard adherent conditions, while the 3D model employed ultra-low attachment plates to promote spontaneous aggregation and spheroid formation over 11 days. Spheroids were monitored microscopically for structural integrity, and both tumor tissues and spheroids were analyzed via hematoxylin-eosin staining and immunohistochemistry using Vimentin and Melan-A markers to confirm phenotypic conservation. Cytotoxicity was assessed in 2D models using the MTT assay and in 3D models with a fluorescent viability kit (Calcein-AM, propidium iodide, Hoechst 33342), applying a range of concentrations for both drugs. The effectiveness of doxorubicin and vincristine was compared, and a methyl methanesulfonate (MMS) control was used for assay benchmarking. Results showed that doxorubicin exhibited strong cytotoxic effects in both 2D and 3D models. In monolayer cultures, cell viability decreased to $18.3 \pm 2.1\%$ at $10 \mu\text{g/mL}$ of doxorubicin, while in 3D spheroids, the same concentration resulted in $42.7 \pm 3.4\%$ viability. In contrast, vincristine achieved a maximal reduction to $33.5 \pm 2.9\%$ viability in 2D and $61.2 \pm 4.1\%$ in 3D at $20 \mu\text{g/mL}$. Control treatment with MMS ($100 \mu\text{M}$) showed only partial cytotoxicity in 2D (viability $\sim 39.6 \pm 2.8\%$), suggesting inherent chemoresistance of the melanoma-derived cells. Doxorubicin elicited a stronger cytotoxic effect than vincristine across both systems, although the spheroids displayed increased resistance, consistent with *in vivo* tumor behavior. The 3D spheroid models successfully recapitulated essential histoarchitecture and cellular heterogeneity of the original melanoma, including the presence of necrotic cores and melanocytic marker expression. These results highlight the preserved tumor-like behavior of the 3D cultures and their enhanced predictive value over conventional 2D assays. These findings underscore the relevance of 3D models for drug screening, as they better reflect *in vivo* tumor microenvironments and therapeutic barriers. In conclusion, the integration of canine derived spheroids into veterinary



preclinical pipelines offers a promising alternative to traditional *in vivo* models, aligning with the 3Rs principles. This platform enhances the predictive power of cytotoxicity assessments and supports the advancement of precision medicine in companion animals through personalized therapeutic selection based on *ex vivo* tumor profiling.

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Animal welfare and humane endpoint assessment in animal models for endocrine disruptor studies

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The 3Rs have been established globally as guidelines to implement alternatives that improve the well-being of animals used in testing and research studies. While the 3Rs guidelines are extensively recognized for guaranteeing ethical and humane treatment of animals in research, they are still not accepted throughout all life science areas. Endocrine disruptor bisphenol A is one of the most chemical products used in a wide industry applications. Multiple investigations have shown reproductive toxicity, cytotoxicity, genotoxicity and oxidative stress induced by BPA in *in vitro* and in *in vivo* models. The understanding of the application of 3Rs principles in endocrine disruptor studies needs an adequate experimental design and an appropriate animal welfare assessment. Its known that gerbil model help to develop the field of biomedical research and contribute for the development and testing of vaccines, drugs and surgical techniques used in human and veterinary medicine. However, increased attention to the welfare of the animals used have gained growing awareness of animal rights and ethical issues. In this investigation, we carry out toxicological, physiopathological, behavioral and reproductive studies in gerbil model. Specifically, the reproductive system is an excellent model to conduct the study of xenohormone dose effects on the assessment of endocrine-sensitive physiological and behavioral endpoints. Additionally, we will focus on useful practical refinements to prevent or reduce suffering, and we will describe the use requirements of gerbil model in the endocrine disruptors research. Enhanced consciousness and insighting of the specific endocrine-metabolic properties of gerbil breeding conditions and housing practices can improve study results and support reproducibility. The present study indicated the necessity for improved testing methods for endocrine disruptor chemicals, the importance of reducing exposure and susceptibility to disease, and suggested the necessity for specific assessment methods to be employed to improve human and animal health.

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Advancing non-animal methods: Corneal microphysiological models for assessing air pollutant-induced ocular damage

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Particulate matter (PM), particularly fine (PM_{2.5}) and coarse (PM₁₀) fractions, has been associated with ocular surface disorders, including irritation, inflammation, and oxidative damage. This study aimed to evaluate the toxicological effects of PM on corneal function using *in vitro* microphysiological systems (MPS), contributing to the development of animal-free models for ocular toxicity assessment. Airborne particulate matter (PM) was collected over a 30-day period from air filtration units installed in public transportation vehicles in downtown Medellín, Colombia. Coarse particulate matter (CPM) was retained using commercial filters with ISO16890 classification ($\geq 50\%$ efficiency for PM₁₀), while fine particulate matter (FPM) was captured by integrating electrospun nanofiber membranes over these filters, achieving up to 65% efficiency for PM_{2.5}. Two experimental *in vitro* models were employed: a 2D monolayer of rabbit corneal epithelial cells (SIRC, ATCC® CCL-60™) cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and a 3D corneal microphysiological system (MPS) consisting of co-cultures with mouse fibroblasts (BALB/3T3 clone A31) seeded as a basal layer and overlaid with SIRC epithelial cells to simulate the architecture of the corneal tissue. Ocular irritation potential was assessed following OECD Test Guideline 491 (Short Time Exposure, STE), by exposing SIRC monolayers to two concentrations of each PM fraction (0.05% and 5%) for 5 minutes, followed by MTT viability assays and classification according to the Globally Harmonized System (GHS). Ocular corrosion was evaluated in the MPS by exposing cultures to increasing concentrations (1, 2, and 5 mg/mL) of PM for 24 hours. Subsequently, one set was immediately tested for viability (MTT), and the second set was incubated for an additional 24 hours in fresh medium to evaluate potential for recovery. Genotoxic effects were assessed in SIRC monolayers via the alkaline comet assay after 1 hour of exposure to PM at 1 and 2 mg/mL. In parallel, expression of inflammation- and oxidative stress-related genes (IL1 β , SOD1, GPX1) was quantified by RT-qPCR using RNA extracted from treated SIRC cultures. The reactions were normalized to ACTB and analyzed by the $\Delta\Delta C_t$ method. All experiments were conducted in triplicate, and statistical significance was determined via ANOVA or t-tests as appropriate ($p < 0.05$). STE results classified CPM as a Category 1 irritant, while FPM was not categorized. In the MPS, FPM caused



a dose-dependent decrease in viability (down to 8.7% at 5 mg/mL), along with marked upregulation of IL1 β and downregulation of SOD1 and GPX1. The comet assay confirmed increased DNA fragmentation. CPM showed milder responses and partial recovery post-exposure. The *in vitro* MPS developed here successfully model corneal responses to airborne pollutants. These systems represent ethical, predictive, and reproducible alternatives to animal testing, supporting the implementation of the 3Rs in ocular toxicology.

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A-STAAAR project introduction: Model-specific strategies for standardizing neurotoxicity testing using zebrafish and *C. elegans*

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Traditional toxicity testing has heavily relied on mammalian models such as rodents. However, concerns over cost, ethics, and limited human relevance have increased interest in alternative approaches. As model organisms, zebrafish (*Danio rerio*) and roundworm (*Caenorhabditis elegans*) are genetically tractable, rapid, and predictive models for

neurotoxicity evaluation. Despite their advantages, standardized test methods for these models remain underdeveloped. This project aims to develop and propose standardized testing strategies using *D. rerio* and *C. elegans* to support regulatory acceptance in both developmental and degenerative neurotoxicity.

We are developing standard operating procedures (SOPs) for each model. For zebrafish, test protocol is being aligned with the OECD IVB batteries, particularly focusing on endpoints for which “ToxTemps” (OECD toxicological testing templates) have not yet been validated. Key endpoints include NPC proliferation, cell migration, neurite outgrowth, and myelination. Among these, myelination is considered a highly feasible zebrafish-specific endpoint with strong potential for regulatory acceptance. For *C. elegans*, neurotoxicity endpoints include oxidative stress, neuronal morphology, locomotor activity, and behavioral responses. In addition, alternative animal models are being developed to study neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) and Parkinson’s Disease, which play a key role in evaluating the efficacy of pharmaceutical substances. For mechanistic evaluation, analyzing organelle function and performing neurochemical profiling based on LC-MS/MS are also being conducted. Various reference substances are being applied to optimize test conditions.

These model-specific strategies are designed to complement existing mammalian-based guidelines and fill critical gaps in alternative neurotoxicity testing. Zebrafish is used to assess developmental neurotoxicity endpoints, while *C. elegans* focuses on degenerative mechanisms. This project enhances test reliability and prepares for regulatory acceptance by OECD and ISO through the establishment of scientifically valid endpoints and the development of SOPs and QC protocols.

The outcomes of this project are expected to propose new OECD/ISO test guidelines and accelerate the global adoptions of standardized alternative neurotoxicity testing strategies.

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Suspension-grown 3D colonoids recapitulate human colonic epithelial drug transport, metabolism, and distribution *in vitro*

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In next generation risk assessment, there is a pressing need for human relevant *in vitro* models of the intestine and other barrier organs. Our objective was to study absorption, distribution, metabolism, and excretion (ADME) of xenobiotics in a physiologically relevant colonic *in vitro* model. To this end, we aimed to establish three-dimensional (3D) colonoids from adult colonic stem cells.

Having established this *in vitro* model, growth in suspension yielded apical- and basal-out 3D colonoids. Both types were subsequently validated using different microscopic and proteomic techniques combined with functional activity assays, using matched patient tissue and isolated primary cells for comparison. Finally, to better understand drug distribution in the colon compartment, we determined intracellular bioavailability (Fic) in colonoids using a representative drug panel. These values were benchmarked against the current standard model for Fic, Human Embryonic Kidney (HEK293) cells.

Colonoid morphology, barriers, and ADME proteomes were characterized and found to be comparable to those of native epithelium, best represented by primary cell isolates. Key transporters (PGP, BCRP) and phase I (CYP3A) and II (SULT1A, and UGT1A families) drug metabolizing enzymes (DMEs) were detected via orthogonal methods (global and targeted proteomics, immunocytochemistry staining) and functionally characterized. Notably, colonoid phospholipid content and drug accumulation kinetics differed from that of HEK293 cells, most strongly impacting Fic of polar compounds.

We conclude that colonoids are a physiologically relevant *in vitro* ADME model. Importantly, simpler models such as conventional monolayers lack the 3D geometry and *in-vivo*-like lipid and protein profiles displayed by the colonoids which are needed to recapitulate colonic epithelial ADME.

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Evaluation of nanotoxicity using dechorionated zebrafish embryos

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Zebrafish embryos are emerging as an alternative model; however, their use may be limited by the restricted direct contact between the embryos and nanomaterials. In 2020, the International Organization for Standardization (ISO) developed a new standard test method, ISO/TS 22082:2020, using dechorionated zebrafish embryos. This study evaluated nanotoxicity using ISO/TS 22082:2020. First, we observed NM uptake into embryonic bodies using 50-nm fluorescein 5(6)-isothiocyanate-incorporated silica nanoparticles. Second, we investigated the physiological effects of chorion removal using pronase on zebrafish embryos. Finally, we evaluated the nanotoxicity of metal oxide and carbon-based NMs using ISO/TS 22082:2020. Results confirmed limited transportation of NMs into embryonic bodies by the chorion. Dechorionated zebrafish embryos exhibited no negative physiological effects. Nanotoxicity in dechorionated embryos was more sensitive than in intact zebrafish embryos. To our knowledge, this is the first study evaluating NM toxicity using the new standardized test method, ISO/TS 22082:2020, and could increase the utility of dechorionated embryos as an alternative model for nanotoxicity evaluation.

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In vitro metabolism and permeability models to estimate human fraction absorbed after oral dosing

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The determination of human exposure via the oral route usually begins with measurements of the permeability of the compound in question over monolayers of intestinal epithelial cells. Permeability is commonly measured in the Caco-2 cell model. Caco-2 cells are, however, metabolically incompetent in that they do not express the CYP family enzymes responsible for phase I intestinal (pre-systemic) metabolism. In accordance with this we see very little or no metabolism for many of the WP3 molecules studied in Caco-2 cells. We are therefore isolating primary human jejunal enterocytes and establishing 3D enteroid models derived from human jejunal stem cells as a better mimic of the human jejunal enterocytes *in vivo*. Primary human enterocytes show detectable expression and activity of CYP and Phase II enzymes. Incorporating metabolic data generated from enterocytes into PBPK models generated plasma concentration profiles in good agreement with human clinical data. The enteroids show an *in vivo*-like expression of tight junction proteins and CYP enzymes, phase 2 enzymes and transporters involved in uptake and efflux in the intestinal epithelium. The enteroids form an epithelial barrier excluding hydrophilic probe molecules of various sizes, exhibit active transport processes and form drug metabolites. Together, these *in vitro* models should provide tools for assessment of human exposure of drugs and xenobiotics.

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Cardiac safety evaluation of echinchrome A using human cardiac organoids

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Cardiovascular diseases are among the leading causes of mortality worldwide, frequently triggered by the cardiotoxic effects of environmental chemicals like particulate matter, arsenic, and various pharmaceuticals. There exists a critical need for innovative preclinical models that accurately predict cardiac toxicity. This study introduces stem cell-derived cardiac organoids (hCOs) as an advanced preclinical model that offers a three-dimensional structure and a physiological environment closely resembling the human cardiac system. This model addresses the limitations of traditional 2D cell cultures and mitigates ethical concerns associated with animal testing. Through molecular characterization, we confirmed that hCOs replicate human cardiac functionalities. The hCOs, characterized by expression of key cardiac markers (cTnT, CX43) and functional ion channels (Cav1.2, Nav1.5, hERG), exhibited structural and electrophysiological properties reflective of human cardiac physiology. Evaluating cardiac safety is crucial in determining the success or failure of drug development. To assess the cardiotoxicity of Echinchrome A (EchA), a therapeutic agent for ischemic heart conditions, we employed stem cell-derived cardiac organoids as a preclinical model. Using multi-electrode array (MEA) analysis, we assessed the effects of EchA at concentrations ranging from 0.1 to 30 μ M on electrophysiological parameters, including beat period, field potential amplitude, field potential duration, and spike slope. EchA treatment showed no significant changes in these parameters, indicating its non-toxic electrophysiological profile. Cellular viability and lactate dehydrogenase (LDH) assays revealed no cytotoxic effects of EchA across tested concentrations. Contractility assays demonstrated that EchA did not alter contraction or relaxation velocity, nor the time to 50% maximal contraction and relaxation. These findings underscore the utility of cardiac organoids as a robust platform for assessing the safety of drugs and environmental chemicals, potentially revolutionizing cardiac safety pharmacology by bridging the gap between conventional models and human biological responses.

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Refinement strategy in laboratory animal science: Molecular detection of murine norovirus (MNV), murine reovirus (MuReoV) and murine astrovirus (MuAstV) in mice from SPF and conventional animal facilities

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In laboratory animal science, refinement strategies are essential to minimize animal suffering and improve scientific outcomes. Health monitoring plays a key role in identifying infectious agents that may compromise research validity or animal welfare. Conventional methods often require animal sacrifice or are limited to postmortem analyses, with low sensitivity for subclinical infections. Molecular diagnostics offer a more humane and effective alternative. This study applied molecular tools to detect Murine Norovirus (MNV), Murine Reovirus (MuReoV), and Murine Astrovirus (MuAstV) in fecal samples from 297 clinically healthy mice (*Mus musculus*) housed in SPF and conventional facilities. Fecal samples were collected non-invasively directly from the cages, avoiding animal handling. Viral genome was extracted using the Purelink[®] viral RNA/DNA kit from a 10% fecal suspension prepared with PBS buffer. The extracted RNA was reverse transcribed using the Applied Biosystems[™] High-Capacity cDNA Reverse Transcription Kit, and the resulting cDNA was used in qPCR assays with TaqMan probes (TaqMan Master Mix Kit, Invitrogen[®]), conducted on the Applied Biosystems StepOne system. To standardize the technique, a synthetic oligonucleotide containing target sequences for all three viruses was constructed. A second synthetic oligonucleotide containing the murine GAPDH gene was used as an internal control for extraction and reaction performance. The target regions amplified were: ORF1 for MNV and MuReoV, ORF1b for MuAstV, and GAPDH for *Mus musculus*. Molecular screening revealed an overall positivity rate of 29%, with marked differences between housing types: 21.6% in SPF animals and 57.4% in conventional animals. Among the positive cases, 47.7% were positive for MuAstV, 27.9% for MNV, and 24.4% for MuReoV. MuAstV was particularly prevalent in younger animals, possibly due to its emerging nature. Results indicate that environmental and husbandry factors influence viral presence more than genetic background or individual immune status. These findings highlight the value of molecular tools as a refinement approach aligned with the 3Rs principle, reducing the use of animals by avoiding unnecessary repetition of experiments compromised by undetected infections. Molecular surveillance increases detection accuracy without requiring euthanasia and improves reproducibility by ensuring knowledge of the animals' microbiological status. By allowing early intervention and reducing disease burden, PCR-based monitoring promotes animal welfare without compromising scientific integrity. We propose that regular molecular diagnostics be adopted as part of routine health programs in animal facilities, aiming for early detection of viral agents, minimization of suffering, and reduction of experimental variability caused by latent infections.

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French Center for 3Rs: Actions toward replacement and reduction

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The French Center for 3R (FC3R) has for a mission to encourage and implement the Replacement, Reduction, and Refinement (3Rs principle) of animal experimentation in France through the promotion of responsible and innovative research, education and transparent communication. The FC3R's dedication to promote and advance alternative methods in life sciences research results in a great interest in microphysiological (MPS) systems.

One of the FC3R's core missions is the provision of research funding: our calls for projects already bolstered the development and application of MPS in life sciences with a high-impact for the 3R, as they allow to Replace – completely or partially – the use of animals and/or animal products in laboratories.

Considering the importance of knowledge dissemination and training in offering researchers access to the latest models and methodologies, the FC3R intensively communicates about innovation, grants, awards, evolution of practices and regulations; and recognizes through interviews, conferences and awards researchers whose accomplishments lead forward the 3Rs in France.

In order to map the scientific knowledge and facilitate scientific collaborations, the FC3R has developed AREA, a directory of the use of alternative methods – including cell culture approaches – in France.

The FC3R also advocates for Open Science and collaborative efforts by providing a platform for unpublished-data sharing "Short Notes", and a catalogue of international training offers. Moreover, the FC3R is willing to accompany researchers with their experimental design through project engineering support, in order to empower scientists to harness MPS for scientific breakthroughs, while ensuring that funded research projects are effectively executed and translated into impactful outcomes.

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Genomic surveillance of murine enteric viruses as a refinement tool in animal experimentation

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The detection and genomic characterization of murine pathogens is a powerful tool for enhancing health surveillance in animal facilities and represents an alternative to the direct use of sentinel animals. In this study, we employed molecular epidemiology and evolutionary inference approaches to investigate the genetic diversity of Murine Norovirus (MNV) and Murine Astrovirus (MuAstV), highly prevalent enteric viruses with potential impact on experimental models. A total of 297 fecal samples from mice (*Mus musculus*) were analyzed by RT-PCR, followed by sequencing of partial fragments of the viral RNA polymerase gene. The obtained sequences were aligned and subjected to phylogenetic analysis using Bayesian inference with the BEAST software, applying nucleotide substitution models and a molecular clock to estimate evolutionary relationships and divergence times among the detected strains. For MNV, high genetic variability was observed, with the sequences clustering into different clades related to previously described genotypes (such as MNV-1 and CR6). Bayesian analysis indicated multiple introduction events and suggested the persistence of genetically distinct variants over time, revealing complex dynamics of intra- and inter-colony viral dissemination. In contrast, MuAstV showed low intraspecies diversity, with most sequences forming a single clade, indicating the circulation of a dominant lineage, possibly associated with its recent emergence as a pathogen in laboratory animal facilities. The phylogenetic and temporal analyses allowed the identification of genetic clusters and estimates of local circulation time of the variants, with the potential to map routes of introduction and persistence of these agents in experimental environments. These data are essential for informed decisions regarding quarantine, colony disposal, or rederivation, preventing the exposure of animals to subclinical infections that could compromise scientific protocols. The application of genomic and advanced statistical tools in microbiological surveillance allows for a reduction in the use of sentinel animals and minimizes experimental losses caused by uncontrolled biological variability. By integrating phylogenetic and Bayesian analyses into routine monitoring, it becomes possible to anticipate health risks with greater precision, replacing invasive practices and promoting reproducible science. Thus, this study highlights the role of genomic surveillance as a promising alternative method within the 3Rs framework, particularly in the refinement axis and potentially in the replacement of animal use for microbiological diagnostics in animal facilities.

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Launch of the DIM testing project: When animal testing is difficult, impossible and meaningless (DIM) and implications for REACH Regulation

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The EU's REACH Regulation mandates that animal testing should be used only as a last resort, as outlined in Article 25. Adaptations of this standard (i.e., using animal test models) testing regime are operationalized primarily through Annex XI. Despite this, traditional *in vivo* testing remains common, often due to procedural rigidity and/or lack of understanding or proper application of regulatory framework, rather than scientific necessity. Despite REACH's adaptive mechanisms, including exposure-driven adaptations and the use of *in silico/in vitro* methods, these are frequently underutilized or inconsistently accepted. Approximately 5-7 million animals have been used or are in use for new *in vivo* toxicological and ecotoxicological studies under REACH (Knight et al., 2023, PMID: 37470350). Annex XI offers theoretically robust pathways to avoid animal tests. Yet ambiguities throughout the adaptations to information requirements (Column 2) in the REACH Regulation, the respective regulatory guidance documents, and the draft decision requests can lead to differences in interpretation, and to the dominance of prescriptive data requirements, hindering its effective application.

The AFSA Collaboration launched a multi-phase project to address persistent misalignments between regulatory expectations and scientific best practices regarding non-animal approaches, with a focus on animal tests that are Difficult, Impossible, or Meaningless (DIM). DIM testing refers to cases where *in vivo* studies have been performed or requested when they are scientifically unfit, unjustified, technically infeasible, or irrelevant to real-world safety outcomes. Such tests often yield redundant, unreliable or unusable results, particularly for substances with properties that compromise standard protocols, such as volatility, insolubility, or rapid degradation. Moreover, these unnecessary tests can have a significant negative impact on animal welfare, subjecting animals to distressing and painful procedures without generating meaningful scientific data.



The AFSA DIM Testing Project plans to systematically review registration dossiers and compliance decisions to quantify the prevalence of DIM tests and identify missed opportunities for scientifically sound, non-animal alternatives.

The multidisciplinary expert group has defined objective DIM criteria for substances (to be) tested and applied them to case studies across sectors, revealing recurring gaps in guidance clarity, methodological relevance, and procedural flexibility. Preliminary findings suggest a notable reliance on animal testing in scenarios where adaptations or modern methods would have been sufficient. Initial evidence suggests that ecotoxicological endpoints seem to be most affected, with high animal use and limited regulatory value. Based on these insights, the project has formulated preliminary recommendations to improve the implementation of the last resort requirement, increase regulatory acceptance of novel methods, and clarify when standard animal tests are inappropriate.

As the European Commission prepares the REACH revision, this work highlights a timely opportunity to strengthen enforcement of Art. 25, modernize testing frameworks, and uphold REACH's ethical and scientific commitments. A regulatory shift toward relevance-based, exposure-led safety assessment, rooted in robust non-animal methods, is essential for reducing unnecessary testing while maintaining high standards for human and environmental protection.

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A methodological approach for animal testing refinement validation using retrospective data analysis

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Implementing refinements in animal testing protocols presents significant validation challenges that often necessitate additional experimental studies. We propose a methodological framework utilizing previously collected testing data to validate time-based refinements without additional animal experimentation. This systematic approach, informed by established statistical validation principles in the scientific literature, serves as an analytical pathway for evidence-based refinement implementation. This methodology includes a detailed analysis of time-based patterns in historical data to find opportunities for refinement. Key analytical components include temporal distribution analysis to quantify outcome timing patterns, parameter stability assessment across different timeframes, and measurement agreement evaluation through correlation coefficients and Bland-Altman analysis. For regulatory testing contexts where pass/fail decisions are critical, the methodology includes categorical classification analysis using sensitivity, specificity, and weighted kappa coefficients to compare classifications between traditional and refined timepoints. This approach was applied by our research group at the National Institute for Quality Control in Health (INCQS) to validate reducing observation periods from 48 to 24 hours in mouse lethality assays for antivenom testing (Pereira-Júnior et al., "Reducing time in lethality assay (LD₅₀) for *Bothrops jararaca* and *Crotalus durissus terrificus* venoms and lethality neutralizing assay (ED₅₀) for their respective antivenoms: A 3Rs-based retrospective data validation," *Toxicol*, 2025). Analysis of 518 quality control assays demonstrated that over 98% of deaths occurred within 24 hours, with concordance correlation coefficients exceeding 0.96 and classification accuracy above 98.5%, validating the refined timeframe without additional animal testing. While initially applied to lethality assays, the framework adapts to various outcome measures including binary endpoints, continuous variables, and ordinal data. The methodology supports comparative analysis between traditional endpoints and potential refined alternatives, enabling objective evaluation of whether the proposed refinement maintains equivalence with established procedures. The framework outlines implementation strategies, including regulatory considerations and phased transition plans for laboratories adopting validated refinements. This analytical approach provides a data-driven pathway to enhance animal welfare while maintaining scientific rigor and regulatory compliance, allowing testing facilities to implement refinements using existing evidence and facilitating the adoption of improved practices in animal testing across diverse domains.

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A new *in vitro* test to estimate antivenom potency against *Bothrops* snake venom

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With the advancements of addressing ethical and animal welfare issues, several standards, guidelines and organizations have emerged with the aim of guiding the use of animals in teaching and experimental research. Thereupon the development of alternative methods to *in vivo* tests, based on the principle of the 3 Rs has been implemented. Within this context, the World Health Organization (WHO) began to encourage the development of alternative methodologies in the production of antivenoms. This study aimed to develop and evaluate an avidity ELISA as an alternative test to the *in vivo* lethality neutralizing assay in the production of *Bothrops* antivenom.

Twenty-nine serum samples from horses immunized with the *Bothrops* pool (*B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni* and *B. neuwiedi*) were used, 14 samples from the Fundação Ezequiel Dias (Funed) and 15 samples from the Centro de Produção e Pesquisa de Imunobiológicos (CPPI). The 96-well ELISA plates were sensitized with crude *Bothrops jararaca* venom. Each sample, diluted 1:16,000, was tested in two triplicates, one triplicate receiving 8 M urea solution as a chaotropic agent and the other without urea. Avidity was defined as the ratio of absorbance measurements without urea/with urea (%). All samples used

were also tested in the *in vivo* neutralizing capacity assay employed in the production of antivenoms at both FUNED and CPPI. In this assay, five Swiss Webster mice were used per serum sample along with only one dilution of the venom calculated based on the Lethal Dose 50% (LD50). The minimum potency defined in this assay for *Bothrops* antivenom (5 mg/mL) was tested. The calculated dose was then incubated with 5 LD50 of *B. jararaca* venom at 37°C for 1 hour. After this period, 0.5 mL of this solution was inoculated intraperitoneally into each of the five mice. Deaths were recorded after 48 hours and the results were defined as positive (potency $\geq$ 5 mg/mL), when one or no deaths occur, and negative (potency $<$ 5 mg/mL), when two or more deaths occur. Student T-test was used for institutions comparison and ROC curve for cutoff estimation.

The mean avidity index of the CPPI group of animals, 79% (95% CI 74-84%), was statistically higher than the mean of the Funed group, 70% (95% CI 61-78%). This difference may be related to the fact that the Funed animals had not been immunized for more than a year as occurred with the animals of CPPI. Among the 29 serum samples analyzed, only three were negative in the *in vivo* test, two from Funed and one from CPPI. The negative samples in the *in vivo* test showed a mean avidity index of 60%, with a minimum of 49% and a maximum of 68%. In the positive samples, the mean avidity was 76%, ranging from 42% to 93%. These results demonstrate a great variability in the avidity index among the samples analyzed, probably due to the differences between the immunization cycles performed in each institution and the individual differences in the immune response of the animals. The ROC curve analysis yielded an area under the curve (AUC) of 0.88 (95% CI 0.75-1) and a cutoff by the Youden index of 72%, with a specificity of 1 and sensitivity of 0.85. Thus, 22 samples would be considered positive, reducing the use of 110 mice.

These findings suggest the possibility of implementing the avidity ELISA before the *in vivo* test, in which samples with avidity above the cutoff would be considered positive and those below the cutoff would be subjected to the confirmatory *in vivo* test. Further studies are needed to validate this new methodology as an alternative test.

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Exploratory analysis for animal reduction in bothropic antivenom potency testing

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Refinement and reduction strategies in animal testing are particularly crucial in areas where complete replacement by *in vitro* alternatives faces significant validation challenges. Antivenom potency testing represents one of these critical domains where, despite ongoing efforts, *in vitro* alternatives have encountered obstacles related to complex venom neutralization mechanisms. In this context, optimizing existing animal models through reduction becomes an important intermediate strategy to improve animal welfare. We investigated the possibility of reducing the number of animals used in bothropic antivenom potency testing from eight to four animals per dilution. This preliminary exploratory analysis employed retrospective data from 10 potency tests randomly selected from tests conducted by INCQS during 2023 and 2024, where animals had been housed in two separate cages (A and B) with four animals each per dilution. As this was a retrospective study, no additional animals were used in this investigation, with the original tests conducted under FIOCRUZ CEUA approval (license LW-16/23). Our methodology involved analyzing three different data configurations: the standard approach using all eight animals per dilution (A + B), and two reduced approaches using only four animals per dilution (cage A or cage B). Potency calculations were performed using Combistats software for all three configurations. Data analysis revealed significant discrepancies in final classifications between methods. While the standard method (A+B) yielded 6 satisfactory results, 2 unsatisfactory and 2 invalid, the analysis of cage A alone produced only 3 satisfactory results, no unsatisfactory and 7 invalid. Similarly, cage B alone resulted in 5 satisfactory, 1 unsatisfactory and 4 invalid results. An increase in invalid tests was observed when the number of animals was reduced, representing a critical obstacle for regulatory validation of this method. A relevant finding was the frequent use of the Spearman-Kärber method by Combistats software in analyses with reduced animal numbers, in contrast to the Probit method predominantly used in analyses with the complete set of animals. Of the 8 valid analyses performed with only 4 animals, 7 (87.5%) used the Spearman-Kärber method. The preliminary analysis indicates that a 50% reduction in animal numbers does not maintain the statistical rigor and test validity required in regulatory and quality control contexts. Results from cages A and B were not concordant with each other, revealing significant variability when using only four animals. Based on the results of this exploratory analysis, our research group is designing a new retrospective study with a larger number of tests, aiming to identify the optimal minimum number of animals per dilution. The new methodological approach proposes gradually reducing the number of animals, removing one animal from each dilution at

a time and verifying result concordance with the standard method. The results of this study and the ongoing investigation may provide concrete evidence for regulatory authorities and laboratories seeking to optimize their testing protocols, promoting advances in the 3Rs principles in the field of antivenom quality control.

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Introducing PEERLOGIC, an AI-supported interactive database for animal-free cell culture protocols

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The majority of *in vitro* experiments rely on the use of Fetal Calf Serum (FCS) and/or Basement Membrane Extracts (BMEs such as Matrigel and Cultrex) in their protocols. The use of FCS and BMEs may limit the physiological relevance of *in vitro* models to human conditions, and their production is associated with significant animal welfare issues. Although the number of animal-free alternatives for FCS and BME is increasing, the substitution and implementation of them remains a challenge for researchers as adapting existing protocols requires thorough validation. To aid researchers with the transition to animal-free media, Peerlogic (<https://peerlogic.org>), a new AI-driven open access database has been developed. Peerlogic allows researchers to upload and share their animal-free protocols (including Materials and Method sections of research papers), where AI will extract the relevant details into Peerlogic. In addition, researchers are able to search Peerlogic for animal-free protocols, share their experiences, add modifications and comment on the use of specific protocols. A rating system has been implemented to aid users in finding successful protocols and which acknowledges the usefulness of protocols uploaded by the users. Peerlogic is a public, non-profit database that works in concert with the FCS-free database (<https://fcs-free.sites.uu.nl/database/>) and BME-free database (<https://bme-free.sites.uu.nl/database/>). We invite researchers to actively contribute to Peerlogic by sharing their animal-free protocols, helping to accelerate the shift away from FCS and BME in cell culture and promoting more ethical and physiologically relevant research practices.

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Presentation: Poster



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Antioxidant potential of plant residues from Brazilian biodiversity: An approach aligned with the 3Rs principle and *in vitro* metabolomics

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The Brazilian biomes Amazon, Cerrado, and Caatinga possess unique biodiversity and are rich in fruits that are essential for the subsistence of traditional communities. However, the production chains of fruits such as açai (*Euterpe oleracea*), tucumã (*Astrocaryum aculeatum*), cupuaçu (*Theobroma grandiflorum*), and Brazil nut (*Bertholletia excelsa*) in Amazonas State, babassu (*Attalea speciosa*) and buriti (*Mauritia flexuosa*) in Maranhão State, and babassu and cashew (*Anacardium occidentale* L) in Piauí State generate large amounts of plant waste that remain underexplored for their bioactive potential. Using chemical and biological methods, this study evaluated the antioxidant potential of residues generated during fruit processing from the Brazilian biomes. This study can help the circular bioeconomy through reduction, reuse, and recycling. The research involved extracting bioactive compounds from plant residues and assessing their antioxidant and cytotoxic potential. Extracts were obtained from the peels of tucumã (TUC) and açai (AÇA), Brazil nut shells (CAS), cupuaçu (CUP), buriti endocarp (END), pericarp (PER), peduncle (PED), and cashew nut testa (CAJ). Antioxidant activity was assessed using ABTS•+ and DPPH assays, and the most promising extracts (AÇA, PER, PED, and CAJ) were tested for cell viability using Caco-2 cell lines via the MTT method. NMR also analyzed these extracts to identify metabolites and investigate affected metabolic pathways, with support from the MetaboAnalyst 6.0 platform. The extracts from açai, cashew nut testa (CAJ), and buriti peduncle (PED) and pericarp (PER) showed the lowest IC₅₀ values in antioxidant assays, indicating greater activity, with CAJ standing out (ABTSIC₅₀: 30 ± 2 µg/mL; DPPHIC₅₀: 26 ± 1 µg/mL), outperforming natural sources like kiwi peel and onion, and commercial antioxidants like ascorbic acid. These extracts also exhibited dose-dependent cytotoxicity in Caco-2 cells, with CAJ being the most cytotoxic (IC₅₀: 1.22 mg/mL), possibly due to its high concentration of phenolic compounds. PER and PED showed intermediate values, while açai was the least cytotoxic (IC₅₀: 3.88 mg/mL). Metabolomic analysis revealed that açai and cashew nut testa extracts acted as antioxidant agents by modulating glutamate and glutathione metabolism, while the control group maintained metabolic homeostasis. Açai extract also modulated energy metabolism, influencing the glucose-alanine cycle and the Warburg effect, possibly due to its richness in polyphenols and flavonoids. The cashew nut testa extract affected glycine, serine, and galactose metabolism – pathways associated with nucleotide and glycoprotein synthesis – with effects attributed to the presence of anacardic acids (m/z 341.21408) and their di- and monounsaturated derivatives (m/z 343.22937 and 345.24527), identified by mass spectrometry. Despite its cytotoxicity, the cashew nut testa extract showed the highest bioactive potential due to its impact on antioxidant and ener-

gy-related pathways, indicating the need for further safety studies. Conversely, the açai and buriti extracts exhibited lower cytotoxicity, making them promising for applications that require antioxidant effects without compromising cell viability. These findings highlight the potential of plant residues from the açai, buriti, and cashew nut production chains for use in medical, pharmaceutical, food, and cosmetic industries in the fight against oxidative stress.

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Combined 2D and 3D cell culture models as a strategy to reduce the use of animals in antitumor compound screening

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Research, development, and production of medicines and vaccines involve complex and multidisciplinary activities that must comply with recommendations and regulatory requirements in the preclinical phase. Testing begins with simple biochemical and cellular assays and progresses to more complex systems, such as cell cultures and animal models. There is an international effort to reduce the use of animals, with a focus on technical advances to build better *in vitro* models, including the use of patient-derived cells, 2D versus 3D cell culture conditions, mini-organ systems, organ-on-a-chip, microfluidic technologies, and bioprinting. Three-dimensional (3D) cell culture in spheroids mimics the architecture of solid tumors, offering alternative methods to animal use and promoting faster and more cost-effective assays to accelerate R&D&I of health-related inputs.

The aim of this work is to establish and standardize 2D versus 3D spheroid cell culture systems on the RPT11M Platform, offering it as a service for efficacy and toxicology testing.

The molecules are tested at a single concentration using cell lines in the 2D model, with cytotoxicity assessment performed by the MTT assay. This stage is intended for early-phase projects (TRL 1 and 2), including the screening of new molecules, development of nanopreparations, and synthesis or guided fractionation. When cell proliferation inhibition reaches at least 30%, the samples are forwarded for IC₅₀ determination and selectivity index evaluation in the previously selected cell lines using the 2D model. At this stage, the projects are already at more advanced maturity levels, covering lead compounds, enriched fractions, isolated actives, and optimized nanopreparations. Samples with positive performance are subsequently evaluated in 3D spheroid models using the forced suspension method, with the goal of complementing cytotoxicity data and then advancing to studies on mechanisms of action.

The results showed significant differences in IC₅₀ values between the 2D and 3D models. Two natural molecules isolated from plants, RPT11M_10330 and RPT11M_10332, were evaluated for MCF7 cell



proliferation in both models. We observed that both compounds were active, inhibiting cell growth, and were more effective than betulinic acid ($IC_{50} = 13.0 \mu M$) and equally effective as doxorubicin ($IC_{50} = 0.91 \mu M$) in the 2D model. The 3D model exhibited greater intrinsic resistance compared to the 2D model, as described in the literature; the Ultra-low attachment spheroid culture environment changed the tumor cellular responses to drugs and mimicked the natural tumor microenvironment better than corresponding 2D cultures since the IC_{50} values of this molecules were higher in the 3D model

Each approach has its own strengths and limitations and should be considered both individually and in an integrated manner.

2D models stand out due to their lower cost, ease of handling, and quick execution, making them particularly useful in early screening and development phases. In contrast, 3D models offer greater structural and functional complexity, yielding results that more closely mimic human physiological conditions.

The combined use of 2D and 3D models in preclinical phases enables a more accurate representation of the *in vivo* environment, generating more robust data. This approach not only accelerates and enhances the drug discovery process but also represents a concrete step toward the progressive replacement of animal use in research.

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Exploring different routes of inoculation in the chicken embryo as an experimental model for pharmacokinetic and toxicity analyses of commercial drugs and lipid and polymeric nanoformulations

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The chicken embryo (CE) has become an important experimental model for pharmacokinetics and toxicity analyses due to its ease of handling, serving as a promising alternative for preclinical testing. However, there is still a scientific gap regarding the standardization of toxicity and pharmacokinetics tests depending on the route

of inoculation in this model. This study aimed to evaluate whether the route of inoculation alters the toxicity of drugs and to verify if different routes of inoculation affect pharmacokinetic parameters in serum and allantoic samples from CE. For toxicity analysis, commercial and nanoformulated forms of norfloxacin, lidocaine, and indomethacin (prepared using lipid-based methods) were tested at five decreasing doses: norfloxacin (200-12.5 $\mu g/CE$), lidocaine (3-0.18 mg/CE), and indomethacin (4-0.25 mg/CE), inoculated at 10 days of development via shell membrane (SM), allantois (AL), chorioallantoic membrane (CAM), and amnion (AM). At 16 days of incubation, mortality, embryo weight, and liver and kidney histological analyses were evaluated. Additionally, the presence of norfloxacin in serum and allantoic fluid after this period was assessed. Pharmacokinetic parameters were analyzed using high-performance liquid chromatography (HPLC) by administering the maximum dose of commercial and nanoformulated norfloxacin via the four routes, with samples taken from serum and allantoic fluid at 30 minutes, 1, 4, and 24 hours post-inoculation. In the toxicity test, no embryonic lethal dose 50 (LD50) was identified for any drug. Norfloxacin did not significantly affect embryo weight. Commercial lidocaine caused an increase in embryonic weight, whereas its nanoformulation did not. Indomethacin significantly reduced embryo weight in both formulations. All histological lesions were associated with nanoformulated drugs administered via the amnion. Norfloxacin induced inflammatory changes in the kidney and degenerative changes in the liver. Lidocaine caused kidney degeneration, while indomethacin led to liver degeneration. Regarding pharmacokinetics, the time to maximum concentration and elimination constant were observed only for the commercial drug. A blood maximum concentration time (C_{max}) of 1 hour for norfloxacin was noted across all administration routes, except for the amnion, which showed a C_{max} at 24 hours post-administration. Interestingly, when we evaluated the C_{max} in the allantoic fluid, the drug was only detectable in embryos treated via the amnion. This study demonstrates differences among routes, with the amniotic route showing a pharmacokinetic distribution characterized by a typical absorption curve with a rise, peak, and decline in both allantois and serum, but with slower distribution. Rapid distribution and elimination in the serum were observed for the other routes, but inoculation via the allantois is unsuitable for pharmacokinetic analysis. Thus, it is likely that drug biotransformation occurs in embryos inoculated at 10 days and evaluated at 11 days of incubation, with slower distribution in the amnion route, explaining the higher toxicity observed. Therefore, the chicken embryo can be used as a pharmacokinetic model through serum analysis, with particularities depending on the route of inoculation.

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Exploring inflammation of infection of chicken embryos before 12 days of embryonic development: A promising experimental model?

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The chicken embryo (CE) model offers a practical, ethical, and physiologically relevant alternative for studying infection and inflammation, providing a bridge between *in vitro* and *in vivo* research while avoiding ethical concerns associated with post-hatch animal models. In previous studies, we identified *Salmonella Pullorum* (SP) as a model for inflammation in CE at a late stage of development. However, to refine the use of the embryo in a more ethical context, we aimed to evaluate CE inoculated at 7 or 9.5 days of embryonic development (DED) with a strain of SP and assessed 24 and 48 hours later as a model for acute inflammation. Embryos were inoculated via the allantoic fluid and monitored for mortality and weight changes. Serum and allantoic fluid samples were collected at 24 and 48 hours post-inoculation for analysis. Quantification of acute phase proteins (Serum Amyloid A, Alpha-1-Acid Glycoprotein, Ovotransferrin), cytokines (IL-4, IL-10), and corticosterone was performed using ELISA. Proteomic profiling of serum samples was conducted by mass spectrometry coupled with liquid chromatography (LC-MS/MS), followed by functional enrichment analysis using PANTHER and KEGG databases. Statistical analyses included comparison of means using the t-test, Tobit mixed-effects modeling for proteomics, and PERMANOVA for multivariate comparisons. Embryos inoculated at 7 and 9.5 DED showed a significant increase in mortality at 48 hours post-inoculation, with odds of death 20-fold ($p = 0.0014$) and 16-fold ($p = 0.0142$) higher, respectively, compared to 24 hours. Our study demonstrates that SP infection in CE triggers an early inflammatory response characterized by increased Serum Amyloid A in the allantoic fluid just in CE inoculated at 9.5 DED. There were decreased IL-10 and corticosterone serum levels in CE inoculated at 9.5 DED. Proteomic analysis identified 1,164 proteins, but only a small portion could be functionally classified due to limited annotation data for *Gallus gallus*. Based on available database information, 28 proteins were linked to pathways, 51 to biological processes, and 45 to molecular functions. The analysis revealed a significant reduction in proteins related to the developmental process in infected embryos. Although no significant weight change was observed in CE inoculated at 7 DED ($p = 0.78$), a trend toward significance was noted at 9.5 DED ($p = 0.0506$). At 4 to 11 DED, the Hypothalamic-Pituitary-Adrenal axis is still immature, and early SP infection may further impair its development, likely reducing corticosterone release in response to stress. Although no significant differences were observed in inflammatory pathways in the serum proteome at 24 hours post-inoculation, a significant decrease in IL-10 levels – a key anti-inflammatory cytokine involved in modulating and resolving inflammation – at 48 hours post-inoculation indicated that the inflammatory response may still be ongoing. This interpretation is further supported by the observed

increase in SAA in the allantoic fluid, a marker of acute inflammation. The lack of changes in inflammatory pathways detected by serum proteomics suggests the response may not have been fully captured, possibly due to limited database annotation or low abundance of key mediators. Altogether, these findings suggest that CE at 24–48 hours post-SP infection is in the early phase of the inflammatory response and support its use as a relevant model for studying early inflammation.

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In vivo and in vitro toxicological effects of *Aesculus hippocastanum* and its derivatives (horse chestnut): A systematic review

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Introduction: Horse chestnut (*Aesculus hippocastanum*) is traditionally used to alleviate chronic venous insufficiency symptoms due to its anti-edematous, anti-inflammatory, and venotonic properties. The main active ingredient, aescin, benefits patients with hemorrhoids or peripheral edema. However, studies on the toxicological safety of horse chestnut and its products are scarce.

Objective: The aim of this systematic review was to summarize the current evidence on the toxicological evaluation of *Aesculus hippocastanum* and its derivatives in order to determine the safety of *in vitro* and *in vivo* use.

Materials and methods: This systematic review was conducted following the PRISMA 2020 recommendations. The study protocol was registered in the Open Science Framework Database, available at the following link: <https://osf.io/srdpe>. The electronic search was performed in five databases and grey literature. Studies that included animal models and *in vitro* techniques (Population) exposed to *Aesculus hippocastanum* and its derivatives (Exposure), and that reported an assessment of toxicological effects (Outcome), were included. Methodological quality was assessed using the ToxRTool.

Results: From 465 identified studies, 12 were included according to the eligibility criteria. Of which, eight were *in vitro* studies. Six of these studies were considered “reliable without restriction” due to good methodological quality, while two *in vitro* studies were considered unreliable. Of the total of 12 studies included, five were *in vivo* studies. Only one was considered “reliable without restriction” with good methodological quality, while four were classified as “not reliable.” Most *in vivo* studies evaluated acute toxicity, with doses ranging from 1 to 6000 mg/kg. The most used *in vitro* method was MTT.

Conclusion: We conclude that there is an urgent need for more robust studies, with better methodological descriptions and experimental rigor, to guarantee data reliability, especially *in vivo* data.

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New approach methodologies (NAMs) for chemical hazard risk assessment of cultivated meat

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Cultivated meat (CM) is a novel food produced using animal cell culture under controlled conditions that can potentially replicate the sensory and nutritional profile of conventional meats (Porto and Berti, 2022). This technology is an alternative way of producing animal protein that can reduce the environmental impacts generated by food systems and support global food security (Tuomisto and Rynänen, 2024). The first regulatory approval of a CM product was in Singapore in 2020; nowadays, other products are approved for commercialization also in the USA and Israel. Because this is a novel and disruptive technology, concerns regarding food safety emerged around the world and have been receiving attention from researchers, companies and multilateral organizations. According to a comprehensive hazard identification published by FAO/WHO (2023), CM potential hazards are mostly like those found in conventionally produced foods, however, some of the inputs used in production – such as chemical substances without a history of use in the food industry and commonly applied in biomedical research – may need to be evaluated for potential adverse effects, and their maximum residue limits must be determined. New Approach Methodologies (NAMs) comprise next-generation risk assessment tools that include *in silico*, *in chemico*, and *in vitro* methods that hold promise for advancing safety assessments of novel foods; however, their effective implementation in the food industry needs efforts by the scientific community and policymakers (Manful, et al., 2023). This study explores the suitability of NAMs in evaluating CM safety. Additionally, we aim to share our experience working on generating data to support the establishment of acceptable and safe levels of Growth Factors (GFs) residues in CM products using Mass Spectrometry-Based Proteomics (an NAM approach) and highlight the barriers for NAMs adoption and broad implementation of these methodologies in low- and middle-income countries. To date, our findings point to a combination of structural, regulatory, economic, and cultural challenges as follows: lack of specialized human resources; need for regulatory harmonization; regulatory rigidity and bureaucracy; regional asymmetries in infrastructure; limited funding for food safety R&D; and technical challenges, including method validation and standardization requirements.

Presentation: Poster

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In vitro toxicological and anti-inflammatory effects of the phytochemical marker coumarin from *Mikania* sp. (Guaco): A systematic review and cytotoxicity in J774A.1 macrophage cell line

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Introduction: The two species of *Mikania* (*M. glomerata* and *M. levigata*), commonly called “guaco” in Brazil, are used in traditional Brazilian medicine to treat diseases such as asthma, bronchitis, relieve coughs and aid in the healing of wounds and eczema. The Brazilian Pharmacopoeia of Phytotherapeutics determines the analysis of coumarin content as the phytochemical marker linked to the quality control of Guaco and its derived products.

Objective: The aim of this study was to summarize the current evidence on the anti-inflammatory and toxic activity of coumarin in the literature, evaluating the association between therapeutic concentrations and toxicity threshold. Based on the concentrations found, the toxicity of coumarin was evaluated *in vitro*.

Material and methods: The systematic review followed the PRISMA 2020 guideline. An electronic search was performed in three databases and grey literature. Studies that conducted *in vitro* techniques exposed to coumarin, and that reported an assessment of toxicological or anti-inflammatory effects were included. The methodological quality was assessed using the ToxRTool. For cytotoxicity evaluation, cells from the murine macrophage cell line J774A.1 were cultured in 96-well microplates and incubated with coumarin at concentrations of 1, 10, 50, 100, 200 and 300 µg/mL for 24 hours. Viability was assessed using the MTT method.

Results: From 396 identified studies, 4 were included according to the eligibility criteria. All studies were considered “reliable without restriction” due to good methodological quality. RAW 264.7 cell line was the most used model. MTT was the most common method to assess cell viability, within a concentration range of 0-500 µg/mL. Two articles determined the anti-inflammatory activity of coumarin through the Nitric Oxide and TNF-α, in addition to IL-6, IL-1β, and PGE2. In the *in vitro* evaluation, coumarin reduced cell viability by 72%, 50%, and 45% when exposed at concentrations of 100, 200, and 300 µg/mL, respectively.

Conclusion: Comprehensive *in vitro* investigations of coumarin are essential for elucidating the pharmacological mechanisms and ensuring the safety of this phytochemical marker. Such studies may significantly contribute to the enhancement of quality control measures for herbal medicines derived from the *Mikania* (guaco) species.

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Toxicological effects of the pesticide 2,4-D in HEK293 cell line

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Introduction: The herbicide 2,4-Dichlorophenoxyacetic Acid (2,4-D) is one of the most widely used agrochemicals, present in over 600 commercial products available on the market. Due to its low cost, 2,4-D is employed for selective weed control in agriculture. Adverse biological effects on human and animal health, such as immunotoxicity, teratogenesis, and endocrine disruption, have been previously reported.

Objective: The aim of this study was to evaluate the toxicity of 2,4-D *in vitro*.

Material and methods: Cells from the human embryonic kidney cell line HEK293 were cultured in 96-well microplates and incubated with 2,4-D at concentrations of 0.1, 1, 10, 100, 500 and 1000 µg/mL for 24 and 48 hours. Viability was assessed using the MTT method. Images have been obtained by optical microscopy to qualitatively assess cell morphology before and after exposure.

Results: Exposure to 2,4-D for 24 hours at concentrations ranging from 0.1 to 500 µg/mL induced cell death rates below 30%, whereas 1000 µg/mL caused over 50% cell death. Prolonged exposure (48 hours) to 0.1-1 µg/mL resulted in low cell death (~30%), while 10-100 µg/mL led to moderate cell death (~40%). Concentrations of 500-1000 µg/mL exhibited the highest toxicity (70% and 85%, respectively). Photographs obtained by optical microscopy corroborate the spectrophotometric data.

Conclusion: Our findings demonstrate that 2,4-D-induced toxicity in HEK293 cells, with concentration- and time-dependent effects.

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Exploring spectral imaging for melanoma analysis using 3D-printed phantoms

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Background and challenges: Melanoma is the deadliest form of skin cancer, and early diagnosis significantly improves prognosis. Therefore, new non-invasive and rapid methods of melanoma detection are highly desirable. In this context, spectral imaging has shown great diagnostic potential. However, the challenge of assessing its effectiveness in this application arises. Here, we propose the development and fabrication of phantoms with melanoma-specific features using 3D-printed molds and 3D bioprinting, aiming for fast preliminary validation of different imaging equipment without the need for human or animal testing.

Materials and methods: Melanoma models were fabricated in three different skin phototypes to reduce racial bias. To mimic both skin and melanoma characteristics, natural biomaterials were used, such as gelatin, gelatin methacrylate (GelMA), and alginate. Synthetic melanin was synthesized using polydopamine (PDA). Phantoms (20 x 20 x 3 mm) and molds were designed using a CAD (computer-aided design) software. The size of PDA nanoparticles was analyzed by nanoparticle tracking analysis (NTA). GelMA + gelatin were used to fabricate the dermis, while alginate + gelatin were used to construct the epidermis and the melanoma lesion. Different concentrations of PDA were used to simulate different skin tones. Firstly, the phantoms were shaped using two different 3D-printed molds: one shaped like a trapezoidal prism, to mimic the melanoma lesion; and another one square-shaped, for both the dermis and epidermis. The dermis was fabricated by pouring 10% (w/v) GelMA + 2% of gelatin into the mold and crosslinking with UV light (2 mW cm⁻²) for 10 min in the presence of 1% Irgacure 2959. Epidermis with PDA and melanoma lesion were fabricated by pouring 5% alginate + 2% of gelatin into the molds and crosslinking in a 1 mol L⁻¹ solution of calcium chloride (CaCl₂) for 10 min (epidermis) and 20 min (melanoma lesion). Phantoms were also fabricated using a 3D bioprinter, to achieve a more precise construction. Rheological and mechanical properties of the biomaterials were analyzed. After fabrication, the color (CIELAB values) of each phantom was also estimated.

Results: PDA nanoparticles had a modal size of 449.8 ± 1.9 nm. Biomaterials composition exhibited pseudoplasticity with non-Newtonian behavior, which allowed precise 3D printing at 25°C. The presence of 0.1 mg mL⁻¹ of PDA did not disrupt biomaterials chains, as shown by storage (G') and loss (G'') modulus, which remained around 1.400 and 170 Pa at 1 rad s⁻¹, respectively. However, 0.3 mg mL⁻¹ of PDA significantly decreased these values to 680 and 78 Pa, being more similar to values found for the whole skin. Mechanical properties were within the range of native skin, with PDA interfering with the crosslinking and



decreasing Young's modulus (73.807 ± 4.356 kPa before PDA addition). Finally, the different phototype phantoms obtained presented the following colors (CIELAB values): (15.953, 4.740, 7.688), (35.564, 6.689, 19.946) and (71.848, 0.399, 12.190). A replicable method for fabricating phantoms that mimic skin tissues of different phototypes and with melanoma lesions was successfully developed. These mimetic tissues can be used for testing different spectral imaging systems for melanoma analysis and detection, avoiding unnecessary preliminary tests in humans and animals. In future studies, other phototypes and melanoma features (e.g., uneven chromophore distribution) can be explored.

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Safety assessment of a porcine placental extracellular matrix hydrogel using the U-SENS and BCOP alternative methods

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The growing demand for alternatives to animal use in toxicological research has driven the development and validation of reliable *in vitro* assays for the safety evaluation of biomedical products. In this study, we assessed the cytotoxic and ocular irritation potential of an experimental hydrogel derived from porcine placental extracellular matrix (ECM), intended for applications in tissue engineering and wound healing.

The ECM was obtained from porcine placentas through decellularization and lyophilization processes, then solubilized and neutralized to formulate the hydrogel. Safety was assessed using two OECD-validated methods: the U-SENS assay (OECD TG 442E) for skin sensitization detection via dendritic cell activation, and the BCOP assay (Bovine Corneal Opacity and Permeability, OECD TG 437) for ocular irritation assessment. In the U-SENS assay, U937 cells were exposed to different concentrations of the hydrogel, and CD86 expression was measured as an activation marker.

The results showed no significant increase in CD86 expression compared to the negative control, indicating a low sensitization potential. In the BCOP assay, isolated bovine corneas were exposed to the hydrogel for 10 minutes, followed by evaluation of corneal opacity and fluorescein permeability. The hydrogel did not induce significant changes in corneal transparency or increased permeability and was classified as "non-irritant" according to the test guidelines.

The findings from both methods indicate that the porcine placental ECM hydrogel presents a safe profile regarding sensitization and ocular irritation potential, supporting its suitability for biomedical applications without the need for animal testing. The combined use of alternative methods such as U-SENS and BCOP proved effective in the preliminary toxicological characterization of complex biomaterials, contributing to the advancement of the 3Rs principles (Replacement, Reduction, and Refinement).

Presentation: Poster

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Microbiome-competent PBK modeling and QIVIVE: A tool to assess the toxicological impact of gut microbial bioactivation

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Xenobiotic biotransformation by the human gut microbiome gives rise to novel metabolites with potentially elevated bioactivity and toxicity, but the physiological relevance of such reactions often remains unclear. Microbiome-competent physiologically-based kinetic (PBK) models have emerged as tools to quantify the toxicokinetics of gut microbial metabolites and thus facilitate their toxicological evaluation. However, the methodology to assess microbial biotransformation reactions is not well standardized, causing low comparability of results between different laboratories.

We thus evaluated the impact of experimental conditions on biotransformation kinetics in anaerobic fecal fermentation assays using a set of chemical probes to profile human gut microbial metabolic capacities. We observed a strong impact of media, dilution and interindividual variability, while sample freezing did not affect chemical metabolism.

Lessons from this assay development were then applied to the development of two microbiome-competent PBK models focusing on a) urolithin A as a beneficial gut metabolite and b) 8-prenylnaringenin as a potential endocrine disruptor that is also formed by gut microbiota. In both cases, high interindividual differences in bioavailable metabolite concentrations could be causally linked to differences in microbiomes. Thus, we now develop strategies to address such individual or population-specific effects in chemical risk assessment.

Presentation: Oral



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A versatile vascularized feto-maternal interface model for high-throughput investigation: An ideal replacement for animal models

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Pregnancy is supported by two feto-maternal interfaces (FMIs): the placenta-decidua basalis and the fetal membrane (amniochorion)-decidua parietalis. Existing microphysiological systems (MPS) designed to model these FMIs often lack decidual vascularization and high-throughput capabilities. To address these limitations, we have developed a novel, vascularized MPS for versatile FMI research. The developed MPS comprises a dynamic flow channel embedded in a fibrin-based matrix with HUVEC and maternal decidual cells, connected via a semipermeable membrane through “click-lock strategy” to a human fetal membrane organoid platform (fetal tissue; amniochorion), enabling decidual vasculature-organoid communication. The 3D-printed “click-lock” mechanism and engineered flow platform system have demonstrated successful fabrication and functionality. Computer Fluid Dynamics (CFD) analysis revealed balanced pressure distribution across the organoid regions, with shear stress modulated by channel diameter and culture media flow rate. HUVECs in the fibrin matrix exhibited sprout formation, creating a vascular network. Decidual cells also demonstrated high viability and stromal cell morphology within the matrix. Organoids, composed of multiple layers of human chorionic trophoblast cells, matrix-embedded amnion mesenchymal cells, and a single layer of amnion epithelial cells, have exhibited viability and proliferation. This platform has recreated the FMI with vascularity and has great potential for pregnancy-related research, allowing drug screening and toxicology studies to be conducted in a high-throughput manner, reducing the use of animal models. It can also be adapted to model a variety of interconnected responses, such as endothelial dysfunction and altered immune cell activity, highlighting its versatility for the study of adverse pregnancy complications.

Presentation: Poster

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3D spheroid models from placenta-isolated CTCs and PTCs for fetal-maternal interface studies

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Three-dimensional (3D) spheroid models have emerged as New Approach Methodologies (NAMs) to overcome the limitations of traditional monolayer cultures, which often fail to recapitulate the complex cell-cell and cell-extracellular matrix interactions found *in vivo*. NAMs are critical for pregnancy research as animal models do not mimic the anatomy nor function of the human feto-maternal interfaces (FMI; placenta and fetal membrane). We established a physiologically relevant 3D model of the fetal-maternal interface for investigating pathologies associated with adverse pregnancy outcomes. Cells isolated from human term, not in labor, placental and fetal membrane tissue were used to generate chorion trophoblast cells (CTCs) and placental trophoblast cells (PTCs) spheroids using the hanging drop method. Experiments were conducted to assess the influence of collagen I (col-I) concentration (0, 25, 50, or 100 µg/mL) and initial cell numbers (5, 25, 50, 100, and 1000 cells per drop) on spheroid formation. 1000 cells per droplet with 100 µg/mL col-I cause cells adhered to the Petri dish surface, preventing spheroid formation. Droplets containing 100 cells or less, with 0 or 25 µg/mL col-I, loosely organized into aggregates by day 3 and formed compact spheroids by day 7. Cell viability, assessed using CellTiter-Glo® 3D, remained high across all conditions. Spheroid diameter increased over time, particularly in the presence of collagen, indicating enhanced cellular proliferation. This 3D spheroid model offers a promising platform for studying FMI biology, inflammatory processes, and pregnancy-related disorders in a more biomimetic, high-throughput environment.

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