23ND INTERNATIONAL CONGRESS

ESTIV

ABSTRACT BOOK

MAASTRICHT 2026

29 June - 2 July

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WELCOME ADDRESS

Dear delegates and colleagues

The 23rd International Congress of the European Society of Toxicology In Vitro (ESTIV 2026) will take place in the vibrant and culturally rich city of Maastricht from June 29 to July 2, 2026. Centered around the inspiring theme, “Ensuring Safety, Advancing Science: Bridging to the Future with NAMs” the congress aims to explore the transformative impact of New Approach Methodologies (NAMs) in ensuring safety, promoting innovation, and shaping the future of toxicology.

The congress will kick off with a pre-congress workshop designed to provide practical insights and foster collaboration across disciplines.

Attendees can expect a robust scientific program featuring cutting-edge research, keynote lectures from leading experts, and dynamic discussions that emphasize the importance of bridging sectors, technologies, and nations to advance non-animal approaches in science.

In keeping with Maastricht’s reputation as a hub of connectivity and collaboration, the congress will also include industry-sponsored sessions, lunch-and-learn events, and networking opportunities, fostering a spirit of partnership among academia, regulatory bodies, and industry. The pre- and post-congress workshops will offer further opportunities to delve deeper into practical applications of NAMs, equipping participants with knowledge and tools to implement innovations in their respective fields.

The picturesque setting of Maastricht, with its iconic bridges symbolizing connection, reflects the congress’s mission to build bridges between scientific disciplines, stakeholders, and societal needs. Participants are encouraged to visit the ESTIV 2026 Congress website for detailed program information, registration details, and updates.

The Congress Committees of ESTIV 2026 warmly invite you to join us in Maastricht as we work together to implement NAMs in the future of toxicology.

Helena Kandárová and Clive Roper

Acknowledgements

The ESTIV 2026 congress would not have been possible without the logistic assistance of the conference organiser Klinkhamer | conferences & events as well as the financial support of the ESTIV supporters and sponsors.

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KEYNOTE SPEAKERS

KEYNOTE SPEAKERS

Monday, 29 June 2026
14:15 - 15:00

KEYNOTE LECTURE I

Dr. Katrin Schütte

Dr Katrin Schütte is a policy officer at the EU Commission's Directorate for the Environment, which she joined in 2014. Her responsibilities cover several aspects of the REACH regulation, the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes and the development of the Commission Roadmap towards phasing out animal testing for chemical safety assessments. She is also a member of the steering committee of EPAA (European Partnership for Alternative Approaches to Animal Testing) and Commission co-chair of several EPAA project teams.

Katrin has a degree in nutrition sciences, a PhD in neurobiology from the University of Heidelberg and is a Diplomate of the American Board of Toxicology (DABT) and European Registered Toxicologist (ERT).

Before joining the Commission, she worked for 15 years as a toxicologist and regulatory affairs manager in the consumer products sector.

EC roadmap towards phasing out animal testing for chemical safety assessments

Katrin Schutte[1]
[1]*European Commission*

In its reply to the European Citizens' Initiative "Safe cruelty-free cosmetics – Commit to a Europe without animal testing" (Communication C(2023) 5041), the Commission committed to the development of a roadmap that will outline milestones and specific actions, to be implemented in the short to longer term, to reduce animal testing and that would be pre-requisites for a transition towards an animal-free regulatory system under relevant pieces of chemical legislation (e.g., REACH, Biocidal Product Regulation, Plant Protection Products Regulation and Human and Veterinary Medicines). The Commission will publish the roadmap in Q2/2026 as a communication accompanied by a staff working document describing more details about the proposed actions. At the ESTIV congress, the Commission can report about the first steps undertaken towards the implementation of the Roadmap actions. This roadmap should serve as a structured action plan to accelerate the transition away from animal testing in chemical safety assessments by setting clear objectives and actionable steps. The roadmap intention is to replace all animal testing, including lower species such as invertebrates, while priority should be given to replace the higher, more sentient species such as vertebrates first. The roadmap outlines the key high-level considerations and policy and change management tools required to allow the transition to conducting chemical safety assessments without the use of animals while maintaining or improving protection of human and animal health and of the environment. The roadmap contains recommendations for the transition to non-animal methods, outlines pathways to better coordinate the identification of regulatory needs and to scale up the development, validation, and implementation of non-animal methods or approaches to meet those needs. Lastly, the roadmap proposes an organisational structure to aid with the implementation of the outlined actions and to foster collaboration among EU Member States as well as internationally.

Tuesday, 30 June 2026
08:30 - 09:00

KEYNOTE LECTURE II

Dr. Kelly Coleman

Kelly Coleman is a Sr. Distinguished Toxicologist, Bakken Fellow, and Technical Fellow at Medtronic where he provides preclinical product development support. He is a U.S. Expert on ISO Technical Committee 194's Working Group 11, which is responsible for the medical device toxicology risk assessment standard (ISO 10993-17), and a U.S. Expert on Working Group 8, which is responsible for the medical device sensitization and irritation testing standards (ISO 10993-10 and ISO 10993-23). Since 2009, he has been involved in NAMs validation projects.
Kelly, who earned his Ph.D. in Toxicology from the University of Minnesota, is a Diplomate of the American Board of Toxicology, a European Registered Toxicologist, a Fellow of the Academy of Toxicological Sciences, and is certified in U.S. regulatory affairs for drugs and medical devices.
In addition, he is a past president of the Society of Toxicology's In Vitro and Alternative Methods and Medical Device and Combination Product specialty sections. In 2024, Kelly was inducted into the College of Fellows of the American Institute for Medical and Biological Engineering.

NAMs and Medical Devices: Challenges & Successes

Kelly Coleman[1]
[1]*Medtronic*

New Approach Methodologies (NAMs) are reshaping medical device biocompatibility, but progress has been uneven across endpoints and regions. This keynote will examine where NAMs have succeeded, where they remain scientifically or regulatorily constrained, and what is still needed to advance the reduction and eventual replacement of animal testing for medical devices. Three high priority areas that illustrate both the promise and current limitations of NAMs in device biocompatibility will be highlighted: in vitro irritation, material mediated pyrogenicity, and in vitro sensitization. Validation studies, ISO 10993 standards, and key regulatory issues will be discussed. The rabbit skin irritation test was mandated for decades to evaluate device biocompatibility. In 2009, a comprehensive effort began that led to the largest validation study in ISO's history. This 12 year program culminated in ISO 10993 23:2021, which requires the use of reconstructed human epidermis (RhE) tissues instead of rabbits. Difficulties, setbacks, the 2025 amendment, and the trajectory of global regulatory acceptance of RhE data will be covered. Since 2006, the rabbit pyrogen test has been required by ISO 10993 11 to screen for material mediated pyrogens (MMPs). A recent comprehensive review convincingly demonstrated that MMPs do not exist in medical devices, prompting revisions to ISO 10993 1 and ISO 10993 11. Details of these revisions will be presented, along with information on the in vitro monocyte activation test as a potential replacement. The guinea pig maximization test (GPMT) has been compulsory for over 50 years to evaluate the sensitization potential of medical devices. To replace the GPMT, ISO/TC 194 Working Group 8 has organized a global validation study of in vitro sensitization assays that is now underway. Specifics of this ongoing effort and its implications for future regulatory practice will be provided.

Wednesday, 1 July 2026
08:30 - 09:00

KEYNOTE LECTURE III

Prof. Liesbet Geris

Liesbet Geris is professor in Biomechanics and Computational Tissue Engineering at the University of Liège and KU Leuven in Belgium, and a member of the Royal Belgian Academie for Sciences and Arts. Together with their clinical and industrial collaborators, Liesbet and her team focus on the development of enabling technologies (in silico & in vitro) for studying skeletal degeneration, regeneration and tissue engineering. Liesbet is scientific coordinator of Prometheus, a musculoskeletal Tissue Engineering platform. Her scientific accolades include having received three prestigious European Research Council grants and an honorary doctorate of the University of Amsterdam. She is executive director of the Virtual Physiological Human Society, an international non-profit organization advocating the use of in silico modeling in healthcare through engagement with the clinical community, policy makers, regulatory agencies and patients. She coordinated the ecosystem building and writing of the roadmap for the Virtual Human Twin initiative. Besides her research work, she is often invited to give public lectures on the challenges of interdisciplinarity in research, women in academia and digital healthcare."

Virtual Human Twins for advancing personalised toxicology

Liesbet Geris[1]
[1]*KU Leuven, U.Liège, VPH society*

The Virtual Human Twin (VHT) initiative provides a transformative framework for advancing personalised toxicology by integrating mechanistic knowledge, multi-scale models, and heterogeneous biomedical data into coherent digital representations of cells, tissues, organs and individuals. By enabling the coupling of physiological, biochemical, and exposure data, VHTs support predictive, patient-specific assessment of toxicological responses beyond traditional population-averaged approaches. As an example in this context, structured physiological maps—formalised representations of interconnected biological processes across organs and scales—play an important role by organising domain knowledge into computable, interoperable frameworks that facilitate model integration and reuse. These maps enable the systematic linking of molecular initiating events to adverse outcome pathways within a whole-body context, thereby enhancing mechanistic interpretability and regulatory relevance. Combined with in silico technologies such as Boolean or agent-based models, they allow for large-scale in silico experimentation, uncertainty quantification, and scenario testing across diverse exposure conditions and patient profiles. Formalizing question-based efforts into a coherent digital infrastructure such as proposed by the European Virtual Human Twin initiative [1], will deliver operability-by-design, enhance sharing and reuse, and accelerate the uptake of these approaches. This will support the identification of susceptible subpopulations, optimisation of risk assessment strategies, and reduction of animal testing. enabling next-generation personalised toxicology, fostering more accurate, transparent, and human-relevant safety assessment paradigms.

Thursday, 2 July 2026
08:30 - 09:00

KEYNOTE LECTURE IV

Dr. Jinhee Choi

Dr. Jinhee Choi is Professor of Environmental Engineering at the University of Seoul and Director of the Chemical Big Data Science AI Research Center. Her research spans ecotoxicology, human toxicology, and computational toxicology, with a focus on integrating mechanistic toxicology and data science to advance New Approach Methodologies (NAMs) and Next-Generation Risk Assessment (NGRA) under a One Health framework. Dr. Choi received her B.S. in Biology and M.S. in Environmental Science from Seoul National University, Republic of Korea, and earned her Ph.D. in Environmental Toxicology from Université Paris-Sud, France, where she was trained as an ecotoxicologist. Her doctoral research linked molecular and physiological biomarkers to higher-level ecological outcomes, an approach that later formed the conceptual groundwork for her research on Adverse Outcome Pathways (AOPs). She subsequently pursued postdoctoral training in human toxicology at a medical school at Seoul National University, focusing on oxidative DNA damage and repair—an unconventional transition at the time that shaped her long-term interest in mechanism-based and non-animal approaches to toxicity assessment. In recent years, her work has focused on explainable AI models for toxicity prediction, chemical mixture risk assessment, and One Health–oriented integration of human and environmental toxicology. She has led multiple national and international projects applying NAMs and NGRA principles to regulatory contexts, including consumer product safety and environmental chemical management. Her research emphasizes transparency, interpretability, and regulatory relevance, aiming to bridge advanced toxicological science with practical decision-making.
She is a former President of the Korean Society of Environmental Toxicology and Health and has also served as an advisory member of the Korean Presidential Advisory Council on Science and Technology, reflecting her leadership at both scientific and national policy levels. Dr. Choi has been recognized as a Highly Cited Researcher in Toxicology (2015–2017)by Clarivate Analytics.
Through her interdisciplinary research trajectory, from ecotoxicology to human toxicology, and from experimental science to data-driven NGRA, Dr. Choi continues to advance a mechanistic, integrative vision of toxicology aligned with One Health and sustainable chemical management.

Beyond the black box: bridging human and environmental safety via AOP-based explainable AI for NGRA

Jinhee Choi[1]
[1]*University of Seoul*

Next-generation risk assessment (NGRA) calls for approaches that are not only predictive, but also mechanistically interpretable, human-relevant, and applicable across diverse biological systems. As New Approach Methodologies (NAMs) continue to transform toxicology, one of the key challenges is moving beyond black-box prediction toward models that can support scientific confidence, regulatory trust, and broader application to both human and environmental safety. In this keynote, I will present our work on adverse outcome pathway (AOP)-based explainable artificial intelligence as a bridge between computational prediction and mechanistic understanding. Built on ToxCast-derived bioactivity data and AOP-informed structures, these models are designed to provide biologically interpretable predictions by linking molecular and pathway-level signals to downstream toxicological outcomes. Rather than treating AI as a standalone prediction engine, this framework uses AOPs as a

common mechanistic language that improves transparency and relevance for decision-making. A central theme of this work is bridging. The same mechanistic logic can be extended across endpoints and species, supporting connections between human toxicity and environmental or ecotoxicological responses. In this sense, explainable AI becomes more than a technical advance: it becomes an enabling framework for One Health-oriented safety assessment, where human and environmental protection are considered together rather than in isolation. More broadly, this approach illustrates how AOPs, big data, and explainable AI can help operationalize NAMs for NGRA. By moving beyond the black box, we can build more transparent, scalable, and bridgeable assessment strategies that support innovation while strengthening confidence in future toxicology. Keywords: Adverse Outcome Pathway, explainable AI, Next-generation risk assessment, New Approach Methodologies, One Health.

ORAL PRESENTATIONS

ALL ORAL PRESENTATIONS

Monday, 29 June 2026
16:30 - 18:30

Session 1: We Know Many of the Barriers to Adoption of NAMs, but What are the Solutions?

Chairs: *Clive Roper (Roper Toxicology Consulting Limited) & Benjamin Harding (Health and Safety Executive)*

O-1-1

The place of law and technology transitions; a case study from chemical risk assessment
ABSTRACT #110

Robert Lee[1], Aleksandra Cavoski[1], Laura Holden[1]
[1]*University of Birmingham*

This paper addresses the problem of adopting non-animal methods to address problems of chemical safety. It follows on from earlier field work which explored the socio-technical barriers to the take up of new approach methodologies, which suggested that barriers lay not always in the science but more often in wider processes (including regulation) and commonly in social acceptance of NAMs(1).In the paper we ask whether the transition to NAMs might be regarded as a 'wicked problem' (2) meaning that it is a complex and multi-faceted problem which is difficult to fully resolve. By exploring the concept of a wicked problem, we are able to frame issues that would require resolution in order to allow us to advance the utilisation of NAMs. In doing so we draw upon further empirical work in which we used Delphi methodology to assist with an expert review of problems of transition. This study builds on work on epistemic communities (3), to allow us to separate out areas of consensus and of conflict in order to begin to set priorities and devise strategies to advance the uptake, regulatory acceptance and accommodation within legislation of NAMs.

O-1-2

A chemical industry case study outlining use and adoption of NAMs and NGRA in an EU REACH context
ABSTRACT #111

Jay Dawick[1]
[1]*Innospec Limited*

Next-Generation Risk Assessment (NGRA) is as an exposure-led, hypothesis-driven approach that integrates new approach methodologies (NAMs) to assure safety without animal testing. There are numerous examples in the scientific literature highlighting NGRA for consumer safety assessment of cosmetic ingredients (Dent et al., 2018; Baltazar et al 2020). However, in this case study, NGRA was applied in an occupational safety context for an exclusive use cosmetic ingredient to avoid animal testing in accordance with EU REACH following compliance check action by the European Chemicals Agency (ECHA). The ingredient has been subject to two completely different and contrasting approaches by the EU REACH registrants to address the compliance check decision with one company (Innospec) adopting an animal-free testing approach and the other companies adopting an animal testing approach. This case study will be used to compare and contrast the two different approaches and their outcome(s) and ultimately highlight how NAMs and NGRA can be used to conduct occupational safety assessments without animal testing under the EU REACH regulation for exclusive use cosmetic ingredients.

O-1-3

Turning science advancements into practice - establishing a new ‘gold standard’
ABSTRACT #88

Donna Macmillan[1], David Allen[1], Erin Hill[1], Beta Montemayor[2], Francesca Rapolla[3]
[1]*International Collaboration on Cosmetics Safety*
[2]*Cosmetics Alliance Canada*
[3]*Cosmetic, Toiletry and Perfumery Association*

Although non-animal New Approach Methodologies (NAMs) and Next Generation Risk Assessment (NGRA) are increasingly recognized within the scientific and regulatory communities, their practical uptake remains limited as many stakeholders continue to rely on traditional methods. Barriers such as regulatory uncertainty, lack of harmonized guidelines, limited training, and institutional inertia contribute to this slow adoption. Facilitating change is challenging and it takes courage to learn to 'do things differently'. To encourage broader uptake, further efforts are needed to build confidence, demonstrate utility, and establish clear pathways for regulatory acceptance. Confidence in NAMs and NGRA can be strengthened through targeted education and training initiatives designed for different stakeholder groups, including regulators, industry professionals, and researchers. Demonstrating their utility is essential and can be achieved through stakeholder co-creation of illustrative case studies in collaborative workshops. Establishing clear and consistent pathways to regulatory acceptance is critical. This can be supported through the development and dissemination of robust guidance documents, such as the International Collaboration on Cosmetics Safety's (ICCS) Best Practice Guidance (BPG) series. These BPGs aim to translate the latest scientific developments into structured, accessible resources that guide users in the generation, assessment, and interpretation of data derived from NAMs to support safety assessments. They reflect the collective expertise of multidisciplinary contributors within ICCS and are intended to advance regulatory confidence by promoting transparency, reproducibility, and consistency in applying approaches that provide equivalent or better protection than traditional approaches. Education and training efforts can accelerate the integration of NAMs and NGRA into regulatory practice by building the knowledge, skills, and confidence needed to support routine application. It is vital these methods are well understood and widely used, to enable the mindset shift towards more advanced and relevant science for safety assessment. Scientific progress is achieved by embracing innovative approaches.

O-1-4

Accelerating regulatory use of NAMs & NGRA: commission roadmap towards phasing out animal testing for chemical safety assessment
ABSTRACT #170

Gavin Maxwell[1], Andrew White[2]
[1]*Unilever*
[2]*Unilever R&D*

The Commission's Roadmap towards phasing out animal testing for chemical safety assessment represents a pivotal shift in European regulatory science. The Roadmap outlines a strategic framework to transition to animal-free chemical safety assessment, aligning with the EU's long-standing commitment of replacement as the ultimate goal. The Roadmap sets out short-, medium- and long-term objectives to support the regulatory uptake of New Approach Methodologies (NAMs) and Next Generation Risk Assessment (NGRA) approaches. This presentation will outline the Roadmap human health safety, environmental safety, and change management priorities and explore how we can align regulatory use of NAMs & NGRA and accelerate confidence-building through cross-sector collaboration. By establishing a structured transition plan, the roadmap provides a clear signal to regulators, industry, researchers and stakeholders that animal-free safety assessment approaches are increasingly ready for regulatory use. In this way, the roadmap serves as a catalyst for paving the way for global acceptance of NAMs & NGRA and the transformation of chemical safety assessment. This talk will also discuss the critical enablers for successful Roadmap implementation, including funding, training, and international cooperation, and highlight key challenges that must be overcome.

O-1-5

Fostering Regulatory Acceptance of NAMs in Support of Medicinal Product Development in the EU
ABSTRACT #469

Sonja Beken[1]
[1]*Federal Agency for Medicines and Health Products*

The European regulatory network has a long-standing commitment towards the application of the principles of Replacement, Reduction and Refinement (3Rs). In this context, the leverage and regulatory acceptance of New Approach Methods (NAMs) 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 integration of NAMs in regulatory testing of human as well as veterinary medicinal products. This presentation will provide a regulatory view on the specific challenges and opportunities related to the regulatory acceptance of NAMs for the testing of medicinal products. Experience gained from past related regulatory discussions (e.g. ICH E14-S7B Q&A, ICH S1B(R1), ICH S5(R3)), 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, will be discussed.

Tuesday, 30 June 2026
09:00 - 10:30

Session 2a: Update to OECD Guidance Document 34 on validation of methods & Defined Approaches

Chairs: *Helena Kandárová (CEM SAS) & Anne Gourmelon (Organisation for Economic Cooperation and Development)*

O-2a-1

Chapter IV: Guidance on the Design and Conduct of Validation Studies
ABSTRACT #2

João Barroso[1], Silvia Casati[1], Effrosyni Katsanou[1], Ingrid Langezaal[1], Milena Mennecozzi[1], Anne Milcamps[1], Pilar Prieto Peraita[1], Lucia Selfa Aspiroz[1], Valérie Zuang[1]
[1]*European Commission, Joint Research Centre*

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]. Since then, various RC templates have been developed, each with similarities and differences in criteria and purpose [2, 3]. The availability of multiple resources and the lack of international standardisation pose a challenge for method developers and regulatory authorities. To address this, the Project Group on the revision of the OECD GD 34, co-led by the European Commission Joint Research Centre (JRC), the US, and the Netherlands, aimed to develop harmonised 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). 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 prioritisation/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. 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.

O-2a-2

Introduction to the revision of OECD guidance document 34 on method validation
ABSTRACT #6

Anne Gourmelon[1]
[1]*Organisation for Economic Cooperation and*

Development

The OECD Guidance Document on the validation and international acceptance of test methods for hazard assessment ("GD 34") is being revised, taking into account the 20 years of experience in the validation of a diversity of methods applied in regulatory chemical safety testing. The modernization of the document will provide clearer criteria to determine when a method is ready for validation, peer-review and Test Guideline development. The document will provide more practical guidance on training to prepare for transferability studies, will reference other recent OECD guidance on good in vitro methods practice and guiding principles on the availability and distribution of protected elements in Test Guidelines. The updated guidance will also address the development of defined approaches, where generally results of in vitro/in silico methods are combined, using a fixed data interpretation procedure (e.g. a fonction, a rule-based procedure) to reach a conclusion of regulatory relevance. A project group has been established with experts from government and research institutes, regulatory bodies, contractual laboratories, validation platforms, industry, statisticians, and animal-welfare organisations from OECD countries. The project is jointly lead by organisations that have a strong interest and/or expertise in the validation process leading to robust methods that can be used globally to generate data that can be mutually accepted across countries for purposes of protecting human health and the environment. The expected timeline for completion of the update is 2027.

O-2a-3

Readiness Assessment, Optimisation, Training and Transferability as Foundations of Successful Validation: Insights from the Update of Guidance Document 34

ABSTRACT #494

Helena Kandárová [1,2]

[1]*Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Science,*
[2]*InVitroTox Consulting, s.r.o.*

The increasing demand for innovative, human-relevant and regulatory-accepted New Approach Methodologies (NAMs) has intensified the need for efficient and scientifically robust validation processes. While considerable attention is often devoted to demonstrating the predictive capacity and reproducibility of new methods, experience has shown that successful validation begins long before formal validation studies are initiated. The ongoing revision of OECD Guidance Document 34 (GD 34) has highlighted the critical importance of readiness assessment as an essential prerequisite for validation. Harmonised readiness criteria for both individual methods and Defined Approaches are being developed to support method developers, validation bodies, funding organisations and regulatory authorities in determining whether a method has reached sufficient maturity for validation. These criteria aim to improve transparency, facilitate prioritisation of validation efforts and reduce the risk of unsuccessful validation studies. Beyond readiness assessment, optimisation, training and transferability represent key determinants of validation success. Method optimisation may continue throughout the validation process as additional scientific knowledge and practical experience are gained. Equally important is the ability to transfer a method reliably between laboratories, ensuring robust inter-laboratory reproducibility and broader regulatory applicability. Successful transferability depends on several factors, including comprehensive standard operating procedures, availability of critical method components, effective data management and interpretation frameworks, access to appropriate equipment and, critically, high-quality training programmes. Experience from international validation studies consistently demonstrates that well-structured training and knowledge transfer significantly enhance successful implementation and reduce variability between laboratories. The revised GD 34 places increased emphasis on these aspects and provides practical recommendations to support future validation activities. This presentation will provide an overview of the evolving concepts of readiness assessment, optimisation, training and transferability within the revised OECD GD 34 framework and will illustrate their importance through examples drawn from validation practice and regulatory experience. The discussion will highlight how these elements

collectively contribute to more efficient validation pathways and ultimately accelerate the regulatory acceptance of innovative non-animal methods.

O-2a-4

Reproducibility evaluation revisited
ABSTRACT #86

Roman Liska[1]
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An essential aim of validation studies in regulatory toxicology is to obtain robust information on test method reproducibility, both within laboratories (WLR) and across laboratories (BLR). Traditionally, reproducibility is assessed using single-value statistics, such as the percentage of concordant classifications in repeated experiments (WLR) or in aggregated results from different laboratories (BLR). While these measures are widely adopted, they have key limitations: they do not account for the uncertainty in the estimates and make cross-study comparisons, particularly of BLR values, problematic. To address these challenges, we propose a probabilistic approach to evaluating reproducibility. This alternative method incorporates uncertainty directly into the assessment, offering a more informative and interpretable measure of test method reliability. The approach is demonstrated using two practical case studies, which highlight how it can reveal insights that conventional summary statistics may obscure. By capturing the inherent variability both within and between laboratories in a probabilistic framework, this method provides a more comprehensive and transparent assessment of repeatability and reproducibility, critical elements for regulatory acceptance and scientific confidence in test methods.
Keywords: Probabilistic approach, Within-laboratory reproducibility, Between-laboratory reproducibility, Test method reliability, Repeatability, Reproducibility.

O-2a-5

Revision of OECD Guidance Document 34, Chapter III: Concepts and Approaches to Validation
ABSTRACT #495

Emily Reinke[1]
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This presentation provides an overview of Chapter III of the revised OECD Guidance Document (GD) 34 on the validation and international acceptance of new or updated test methods for hazard and risk assessment. The chapter introduces new guidance on validation concepts, workflows, and study designs used to evaluate methodologies for regulatory and scientific applications. GD34 applies to a broad range of methodologies, including in vitro, in silico, in vivo, and defined approaches, with the goal of ensuring that methods are scientifically robust, reproducible, relevant, and fit for purpose. Recognizing that validation needs differ across methodology types, the chapter describes two complementary validation workflows: one for individual methodologies and one for multiple methodologies or defined approaches. These workflows are adaptable to the intended use of a methodology and to prospective, retrospective, or combined validation strategies. Key workflow elements include relevance assessment, evaluation of within- and between-laboratory reproducibility, protocol finalization, evidence generation, and independent review. Readiness criteria templates support decision making throughout the process. For defined approaches, validation addresses both component methodologies and the integrated Data Interpretation Procedure (DIP), with particular attention to computational approaches involving artificial intelligence and machine learning. The chapter also outlines performance standards-based validation as a means to evaluate mechanistically similar methodologies using predefined performance criteria and reference chemicals.

Session 2b : VHP4Safety — Next generation safety based on human data

Chairs: *Markéta Dvořáková (National Institute of Public Health) & Anne Kienhuis (National Institute for Public Health and the Environment (RIVM), The Netherlands)*

O-2b-1

The VHP4Safety platform: open and FAIR in silico services for next-generation safety assessment
ABSTRACT #65

Marvin Martens[1], Egon Willighagen[1]
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The Virtual Human Platform for Safety Assessment (VHP4Safety) provides open access to in silico services and toxicological data via a modular, user-oriented platform available at platform.vhp4safety.nl. It is designed to support a wide range of users, from risk assessors in industry and regulatory bodies to academia. The platform offers both Graphical User Interfaces for services and Application Programming Interfaces for automated, reproducible workflows. Platform development follows the principles of open science and FAIR (Findable, Accessible, Interoperable, Reusable) data and software: services are containerized using Docker for portability, annotated with standardized metadata, and accompanied by extensive documentation, tutorials, and example use cases. The infrastructure is built and maintained through coordinated efforts in three in silico work packages focusing on toxicokinetics, toxicodynamics, and building the infrastructure. Computational tools are developed alongside newly generated in vitro data to ensure mechanistic and biological relevance to the project case studies. Current services include QSPR and qAOP models, a PBPK modeling framework, an RNA-seq preprocessing pipeline, molecular docking and site-of-metabolism prediction tools, and semantic access to the AOP-Wiki through a SPARQL endpoint. While services are modular and can operate independently, they are designed with interoperability in mind to enable integration into larger risk assessment pipelines. The integrative potential of the platform is illustrated through three regulatory case studies focused on neurodegeneration, nephrotoxicity, and thyroid-mediated neurodevelopmental toxicity. For example, in the neurodegeneration case study, users can combine the AOP-Wiki RDF knowledge graph with QSAR and molecular docking tools to explore chemical interactions with molecular initiating events, mapping these to adverse outcome pathways. By providing transparent, reusable, and human-relevant computational tools, the VHP4Safety platform supports the transition toward next-generation, animal-free safety assessment.

O-2b-2

Usability and acceptance of NAM-based safety assessment of the virtual human platform
ABSTRACT #81

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The focus of this presentation is on social interactions towards usability acceptance of NAM-based safety assessment using the Virtual Human Platform (VHP). The presentation departs from arguments supported by science and technology studies (STS), which contend that improvements in scientific knowledge and technology applications, alone, are not enough to ensure usability or acceptance of innovations. Diverging understandings emerge in the social processes of constituting new technologies. STS literature shows that usability and acceptance of innovations result from the stabilization of shared understandings of artefacts and their meanings. The platform proposed in the VHP4Safety project serves as an instrument to investigate the social production of digital means for NAM-based safety assessment. In these social processes of production, divergent understandings emerge in the interactions of stakeholders with NAM-based proposed tools. Moreover, the VHP intends to organize tools and integrate them into a generic framework for chemical risk assessment. In this integrational effort, the VHP4Safety project followed a constructivist approach, orienting the process as a safeguard to include the diverse perspectives of multiple stakeholders. To access the formation of shared understandings in this constructive approach, a distinction is made between developers and potential users. A deliberate decision was to prioritize potential users with expert and professional knowledge in chemical risk assessment –in scientific research, regulation, and industry. The shared

understandings of these potential users and developers are identified in assumptions and expectations for what the VHP can be used for. Moreover, these expectations and assumptions can be translated into criteria to assess the implementation of current and future versions of the VHP. Results from this constructive approach show that shared understanding of NAM-based safety assessment ties into practical issues. For example, a knowledge base needs to be provided alongside specific tools to help make sense of the information that specific NAMs provide.

O-2b-3

AOP-guided omics for human-relevant assessment of platinum-induced kidney failure

ABSTRACT #496

Devon A. Barnes, Frank A.M. Redegeld[1], Marcha Verheijen[2], Roos Masereeuw[1]
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Platinum-based chemotherapeutics are essential anticancer agents; however, their clinical application is constrained by nephrotoxicity, particularly injury to the kidney proximal tubule. To enable human-relevant, mechanism-based safety assessment, an adverse outcome pathway (AOP)-guided omics strategy was employed to investigate platinum-induced tubular necrosis as a model for drug-induced kidney injury. Conditionally immortalised human proximal tubule epithelial cells were exposed to sub-cytotoxic concentrations of cisplatin, carboplatin, and oxaliplatin, followed by RNA sequencing and pathway enrichment analysis. Transcriptomic analysis across the three platinum compounds revealed a shared stress and repair-associated response, encompassing pathways related to genotoxic stress, proteostasis, cell-cycle regulation, mitochondrial function, extracellular matrix remodelling, and tissue repair. Interpretation of these molecular changes within the AOP framework refined candidate key events for platinum-induced tubular injury, including proteasome amplification, p53-associated stress signalling, mitochondrial translation responses, extracellular matrix remodelling failure, and suppression of regenerative Hedgehog/Wnt-planar cell polarity signalling. These results demonstrate how data generated using new approach methodologies (NAMs) can be structured into mechanistic knowledge to inform the interpretation of drug-induced kidney function failure in next-generation safety assessment approaches such as VHP4Safety.

Tuesday, 30 June 2026
11:00 - 13:00

Session 3a: International Standards as the Bridge to the Future of Safe Medical Devices

Chairs: *Helena Kandárová (CEM SAS) & Jessica Perrin (PETA Science Consortium International e.V.)*

O-3a-1

Practical challenges to the use of NAMs for medical devices – when state-of-the-art science alone is not enough

ABSTRACT #38

Janine Townsend[1]
[1]*Chorley Consulting*

From Class I to Class III medical devices, the current standards and laws are failing the implementation of New Approach Methodologies (NAM's), including Non-Animal Technologies (NAT's) within the medical device sector. The bare bones defining an 'in vitro' before 'in vivo' route are present in the laws, standards and guidance documents but the practicalities faced by device manufacturers are limiting the successful inclusion of NAMs within the biological evaluation process. Why? Through exploration of the experiences and struggles faced by medical device manufacturers, we will present several very real examples of the challenges faced by the manufacturers as they have attempted to navigate the biological evaluation and regulatory approval of their medical devices.

We will discuss the complexity of the nuance between regulatory 'compliance' and regulatory 'acceptance' and what that means to the key stakeholders and the general acceptance of NAMs today for medical devices. Through these practical examples, we will consider why the current marketing methods are failing the NAMs initiative and explore opportunities to further drive the application of NAMs in the Medical Device Sector. The regulations and standards have talked the talk but perhaps it is now time for them to start walking the walk?

O-3a-2

NAM approach for vaginal irritation assay to evaluate irritation potential of feminine care products and medical devices

ABSTRACT #28

Seyoum Ayehunie[1], Tim Landry[1], Mitchell Klausner[1], Alex Armento[1]
[1]*MatTek Corporation*

Although regulatory frameworks are increasingly trending to the acceptance of non-animal testing for specific applications, the integration of non-animal methods into official guidelines remains slow, often requiring up to a decade or more. This study evaluates a physiologically relevant 3D human in vitro vaginal tissue to be used as a New Approach Methodology (NAM) for assessing vaginal irritation assay. The in vitro model offers a reproducible and human-relevant alternative to the rabbit vaginal irritation (RVI) assay to evaluate feminine care products and medical devices in contact with vaginal mucosa. To support translational relevance, an in vitro and in vivo study was conducted using 14 coded test articles (TAs), including preservatives, contraceptives, solvents, penetration enhancers, antiseptics, and surfactants. TAs were applied topically at 2% concentration over five daily exposures across six days. In vivo irritation was assessed using RVI scoring, while the NAM-based in vitro endpoints included MTT viability, transepithelial electrical resistance (TEER), cytokine release, and histological analysis. Results showed that benzalkonium chloride(BZK), nonoxynol-9 (N9), and SDS were accurately predicted as vaginal irritants by the in vitro model based on decreased viability and TEER (<50%). While BZK was identified as a mild/severe irritant in the RVI assay, the effect of N9 in rabbits was highly variable. To further evaluate the assay method, the EpiVaginal model was reconstructed using cells from four different donors and exposed to 55 TAs commonly found in feminine care products. 6/55 and 5/55 TAs were predicted as irritants by viability assay and TEER, respectively. The remaining TAs were predicted to be non-irritants in all four donors, confirming the model's donor-to-donor reproducibility. In conclusion, the study supports the integration of NAM-based vaginal irritation assessments using the EpiVaginal model for regulatory safety evaluations and reduce animal testing. A dedicated working group has been established to further validate this model.

O-3a-3

In vitro irritation testing for medical devices: validation and regulatory acceptance

ABSTRACT #304

Kelly Coleman[1]
[1]*Medtronic*

The introduction of ISO 10993-23:2021 established in vitro reconstructed human epidermis (RhE) models as the preferred approach for evaluating the irritation potential of medical devices, marking a major shift from traditional rabbit irritation tests. This transition was driven by extensive scientific evidence showing that RhE models—originally validated for neat chemicals under OECD Test Guideline 439—reliably detect irritant chemicals in dilute and complex medical device extracts. A pivotal international round-robin study sponsored by ISO, involving multiple laboratories and two OECD-recognized RhE platforms (EpiDerm and SkinEthic RHE), demonstrated that these models correctly identified nearly all irritant polymer extracts and exhibited high interlaboratory reproducibility. The RhE method showed sensitivity comparable to human patch testing and rabbit intracutaneous tests when detecting strong irritants at low concentrations, supporting its regulatory acceptance as a full replacement for in vivo irritation testing. The ISO 10993-23 standard formalized this evidence by requiring validated in vitro irritation tests before animal testing, aligning with ISO 10993-2 animal-

welfare principles. The standard specifies use of both polar and non-polar extracts, extended exposure durations (18–24 hours) to improve detection of low-level irritants, and a prediction model where ≤50% tissue viability indicates irritancy. The 2025 Amendment to ISO 10993-23:2021 further broadens acceptance by adding two additional OECD-439 RhE models (KeraSkin and LabCyte EPI-MODEL24) that demonstrated equivalent predictive performance in catch-up validation studies, reinforcing global confidence in the robustness of the in vitro approach. Collectively, the validation evidence and evolving regulatory framework confirm that RhE-based irritation testing is a scientifically rigorous, reproducible, and ethically preferable alternative to animal methods. This modernized approach strengthens patient safety assessment and advances the global replacement of in vivo irritation testing in medical device evaluation. This presentation will review the status of in vitro irritation testing of medical devices, focusing on validation and global regulatory acceptance.

O-3a-4

Advancing medical device pyrogen testing through the in vitro monocyte activation test
ABSTRACT #44

Shabnam Solati[1]
[1]*MAT BioTech*

The evaluation of medical device biocompatibility has traditionally relied on animal-based testing methods, raising ethical concerns and regulatory challenges. In response to the global shift toward more humane and scientifically robust alternatives, this presentation explores the implementation of the in vitro Monocyte Activation Test (MAT) for the pyrogen testing of medical devices. The MAT has been integrated in the European Pharmacopoeia's general chapter 2.6.30 Monocyte-Activation Test as an alternative to the rabbit pyrogen test (RPT), and now as its full replacement. It has also been accepted into the U.S. FDA's Medical Device Development Tools program, and is currently under review to qualify as a stand-alone medical device release test when it meets the requirements for biocompatibility and sterility testing. The MAT is ethically favorable and offers enhanced human safety with its higher sensitivity, specificity, and capability to detect all pyrogens. The MAT is also suitable for the detection of pyrogens on the surface of medical devices by direct incubation with human blood cells. Surface contaminants are frequently not soluble, and thus cannot be detected by rinsing the device, yet in vivo, they may cause adverse reactions. The RPT and bacterial endotoxin test are only suitable for testing rinsing solutions that do not reflect the entirety of surface contamination, causing false negative results. This presentation overviews MAT approaches and methods described for drug testing in Ph. Eur. 2.6.30 and projects their application on to the context of medical devices. Through various case studies, methods and criteria – as well as any proposed adaptations – shall be discussed and evaluated. Further, this study will introduce a new generation of PBMC-based MAT, unveiled for the first time, with the highest sensitivity on the market, 100% animal-free medium, simplified read-out, and test duration reduced to within just one working day.

O-3a-5

Chasing phantoms? A case study on replacing the rabbit pyrogen test for the evaluation of material-mediated pyrogens in medical devices
ABSTRACT #53

Lindsey Borton[1], Kelly Coleman[2]
[1]*Gradient*
[2]*Medtronic*

The ISO 10993-1:2018 standard provides a roadmap for conducting a preclinical safety evaluation of medical devices. Many devices need to be evaluated for their ability to produce material-mediated pyrogenicity (MMP). MMP is broadly defined in the ISO 10993-11:2017 standard as a "type of pyrogenicity [that] originates from non-endotoxin related factors." Annex G of the ISO 10993-11:2017 standard lists 24 substances that are allegedly material-mediated pyrogens (MMPs). This annex recommends the use of the rabbit pyrogen test (RPT) to detect the presence of MMPs on devices and does not provide an option to use in vitro or non-testing alternatives. This presentation describes the results of an investigation to determine whether MMPs exist in a medical device context and whether the

RPT could detect their presence if they were on a device. To accomplish these goals, the study evaluated whether the Annex G MMPs are actually pyrogenic, explored whether compounds released from medical devices could exert a hyperthermic response through uncoupling of oxidative phosphorylation (UOP), and reported the results of a recent survey of RPT failures and their root causes. The conclusions of this effort were twofold: (1) MMPs do not exist in a medical device context. A small number of compounds could theoretically exert a hyperthermic effect as UOPs if they were present in high enough quantities on the device, but they could be detected with analytical chemistry and risk assessment. 2) There are no documented examples of RPT failures attributable to the presence of MMPs on the device; rather, endotoxin and test system incompatibility were identified as the primary root causes of RPT failures. The MAT can detect both endotoxin and non-endotoxin and serves as an in vitro means to detect biological contamination. While the non-animal methods are superior, the ISO standards have not caught up.

O-3a-6

Converging efforts for the advancement of new approach methodologies in the current regulatory context: safety assessment of medical devices
ABSTRACT #50

Amanda Ulrey[1]
[1]*Institute for In Vitro Sciences, Inc. (IIVS)*

Background and Objectives: The regulatory context of 2025 provided a renewed support to the New Approach Methodologies (NAMs) towards the reduction of animal testing in preclinical safety studies. The US Food and Drug Administration (FDA) and National Institutes of Health (NIH) launched new initiatives and roadmaps to expand innovative, human-based science while aiming to substantially reduce animal use in research. Considered Class II medical devices in the United States, personal lubricants represent a product class that could benefit from this favorable context regarding their required safety testing. Their registration is primarily governed by the FDA while the International Organization for Standardization (ISO) standards play a significant role in ensuring the quality and safety of these products. Materials and Methods: Converging efforts of the Institute for In Vitro Sciences and PETA Science Consortium International (PSCI) created an Industry Consortium of manufacturers of personal lubricants/vaginal moisturizers and companies interested in the advancement of animal alternatives. This consortium works collaboratively with stakeholders to develop an in vitro testing approach that could be used in place of the Rabbit Vaginal Irritation test in FDA pre-market submissions. Concurrent work is conducted to support this NAM platform based on reconstructed vaginal human tissues (EpiVaginal™, MatTek Corporation) for the safety assessment of the personal lubricants as part of an ISO working group. Results: Existing or newly generated in vitro data for vaginal irritation based on the commercially available human reconstructed vaginal tissue model(s) will be analyzed against existing in vivo rabbit data to develop a prediction model for the safety assessment of these products. Discussion and Conclusion: This type of collaborative work and partnership between industry, regulators and other organizations supporting non-animal testing methodologies is an example of a viable process to support implementation of modern predictive toxicology platforms that can be used to make regulatory decisions.

Session 3b: Development and Regulatory Acceptance of NAMs in Genetic Toxicology

Chairs: *Clive Roper (Roper Toxicology Consulting Limited) & Giel Hendriks (Toxys B.V., The Netherlands)*

O-3b-1

Validation and implementation of the ToxTracker assay for mechanistic genotoxicity assessment
ABSTRACT #19

Giel Hendriks[1]
[1]*Toxys*

ToxTracker is a mammalian cell reporter assay that accurately predicts the genotoxic properties of compounds. By evaluating induction of various reporter genes that play a key role in cellular pathways relevant for genetic toxicology, ToxTracker has the advantage of providing insight into chemical mode-of-action (MoA), thereby discriminating direct-acting genotoxicants from cytotoxic chemicals that induce DNA damage secondarily. ToxTracker contains six different GFP-tagged reporter cell lines that together allow the accurate identification of genotoxic substances and discrimination between induction of DNA damage, oxidative stress and/or protein damage in a single test. To investigate how ToxTracker may complement the standard battery of in vitro genotoxicity assays, a comprehensive interlaboratory validation trial was conducted. The goal of this prospective validation study that was approved by the OECD, was to explore the applicability of ToxTracker for regulatory applications, establish the transferability and reproducibility of the assay and to explore how it can be applied to improve the in vitro genotoxicity testing strategies. In the validation trial, seven laboratories tested 64 genotoxic and non-genotoxic chemicals that together cover a broad spectrum of chemical spaces. The validation trial showed a good within-lab reproducibility (WLR) of 73-98%. The between-lab reproducibility (BLR) of ToxTracker was 83%. The interlaboratory validation confirmed the accuracy of ToxTracker to correctly predict in vivo genotoxicity of compounds with a sensitivity of 87% and a specificity of 90%. 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, ToxTracker is a valuable addition to the battery of genotoxicity assays that is applied for regulatory applications. ToxTracker was recently accepted into the OECD test guideline program.

O-3b-5

Development and regulatory acceptance of the gH2AX/H3 method
ABSTRACT #48

Marc Audebert[1]
[1]*INRAE*

The development of efficient, high-throughput in vitro assays for genotoxicity assessment is a major priority. In addition to hazard identification, quantification of the phosphorylated histones H2AX and H3 biomarkers (□H2AX and pH3) provides insights into the genotoxic mode of action (MoA). In 2023, the Organisation for Economic Co-operation and Development (OECD) approved the development of a Detailed Review Paper and Retrospective Performance Analysis for the in vitro □H2AX/pH3 method, i.e., as a new project in the Test Guidelines Programme workplan. For this purpose, we reviewed 300 studies performed in the last 15 years. In total, 815 chemicals were included in a dataset comprising 41 aneugens (5%), 416 clastogens (51%), 17 aneugen/clastogen (2%) and 341 non-genotoxic chemicals (42%). The dataset effectively covered different MoAs for aneugenic (kinases inhibitors, tubulin stabilizers and destablizers) and clastogenic substances (oxidative stress, bulky DNA adducts, topoisomerase inhibitor, inter-strand crosslink, etc.). Moreover, “irrelevant” positive genotoxins (apoptosis or p53 inducers) were also included. Results from commonly used in vitro genotoxicity tests for each chemical have also been collected. Chemical space analysis of the dataset using multiple descriptors was conducted; it demonstrated similar coverage to the REACH and Drugbank datasets. The performance of the in vitro □H2AX/pH3 method (sensitivity, specificity and accuracy) to detect genotoxicity was calculated and compared to the bacterial Ames (TG471) test, the mammalian cell in vitro mutation tests (TG476 and TG490), and the in vitro micronucleus (TG487) and chromosome aberration (TG473) tests. The analyses demonstrated high predictivity of the γH2AX/pH3 in vitro genotoxicity method for in vitro genotoxicity determination (91%), and a high concordance of the method with the MLA/HPRT and the MN/CA assays (85%). Moreover, the method was able to precisely define the genotoxic MoA.

O-3b-2

Benchmark dose modeling and data visualization of multiplex data using ToxPi
ABSTRACT #20

George Johnson[1], Yusuf Hussien[2]

[1]*Swansea University*
[2]*Dr*

Current approaches in genetic toxicology face significant quantitative challenges in analyzing highly condensed, multiplexed toxicity dose-response data, particularly in preserving raw experimental findings and developing suitable visualization methods for interpretable data representation. Effective visualization tools could enable comparative analysis of multiple chemical compounds simultaneously, facilitating hazard and safety characterization. The Benchmark Dose (BMD) model offers a robust solution by providing a "Point-of-Departure" independent of dose selection, allowing interpolation or extrapolation beyond tested doses—unlike traditional methods such as LOAEL/NOAEL. The BMD approach employs statistical models to derive dose-response relationships, determining the dose corresponding to a predefined benchmark response (BMR) associated with adverse effects. By generating confidence intervals (CIs), including lower (BMDL) and upper (BMDU) bounds, the BMD provides a conservative, statistically rigorous potency metric, enhancing margin-of-safety assessments in genotoxicity testing. BMD analysis has been successfully applied across diverse assays, including the MultiFlow/MicroFlow assay, ToxTracker, TGx-DDI transcriptomic biomarker testing, iScreen image analysis, and in vitro-to-in vivo extrapolation, demonstrating its versatility in potency ranking across multiple biological endpoints. Computational tools such as PROAST utilize four-parameter exponential (Exponential Models 3 and 5) and Hill models to derive dose-response curves, integrating assay-specific BMRs to establish 90% BMD CIs. To further enhance interpretability, visualization platforms like ToxPi transform BMD-derived CIs into comparative ToxPi scores and graphical ToxPi profiling and clustering outputs, enabling direct potency comparisons across chemicals by capturing and translating chemical potency into a robust quantitative/visual figure. This integrated approach advances toxicological research by improving chemical hazard/safety assessment and genotoxicity profiling.

O-3b-3

Advancing genotoxicity assessment for regulatory applications by integrating transcriptomics with BMD and IVIVE approaches ABSTRACT #23

Anouck Thienpont[12], Emmanuel Demuynck[12], Eunnara Cho[3], Andrew Williams[3], Matthew J Meier[3], Carole L Yauk[4], Marc A Beal[5], Davie Cappoen[1], Freddy Van Goethem[2], Vera Rogiers[2], Tamara Vanhaecke[2], Birgit Mertens[1]
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In vitro transcriptomics offers a promising avenue for high-throughput, human-relevant toxicological assessment, yet its regulatory application remains limited due to a lack of standardized methodologies. In the context of genotoxicity testing, transcriptomic biomarkers such as GENOMARK and TGx-DDI support both qualitative and quantitative evaluation of gene expression changes. To enable broader regulatory use, robust approaches for deriving transcriptomic points of departure (tPoDs) and translating them to the in vivo situation are needed. Within this context, we evaluated multiple strategies for calculating tPoDs and employed in vitro to in vivo extrapolation (IVIVE) to estimate administered equivalent doses (AEDs). Human HepaRG cells were exposed for 72 hours to 10 well-characterized in vivo genotoxicants at concentrations up to 50% cytotoxicity. Transcriptomic profiling was conducted using a customized TempO-Seq® human S1500+ gene panel. Both GENOMARK and TGx-DDI biomarkers accurately identified genotoxic responses across all compounds. Benchmark concentration (BMC) modeling was applied to derive genotoxicity-specific (tPoD_biomarkers) and generic (tPoD_S1500+) transcriptomic PoDs. High-throughput toxicokinetic (HTTK) modeling was used to convert these tPoDs into AEDs, which

were then compared with (a) previously published AEDs from other New Approach Methodologies (NAMs) and (b) in vivo PoDs from animal studies. In general, generic transcriptomic AEDs were more conservative than those derived from genotoxicity-specific biomarkers. For six out of nine genotoxicants, transcriptomic AEDs were lower than the corresponding in vivo PoDs, suggesting a protective margin. Refinement of kinetic models may further enhance predictive accuracy. These findings support that in vitro transcriptomic data in HepaRG cells provide protective estimates of in vivo genotoxic concentrations, contributing to the advancement of NAMs in regulatory toxicology.

O-3b-4

Single-molecule mutation sequencing (SMM-seq): ultra-sensitive genotoxicity profiling at single-nucleotide resolution ABSTRACT #25

Sam Sharifi[1], Alexander Maslov[2], Luis Popoca[1], Chrisopher De Solis[1], Christopher Bradley[1], Jan Vijg[23]
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Single-Molecule Mutation sequencing (SMM-seq) is an error-corrected next-generation sequencing platform that extends mutagenicity testing from phenotype-based surrogates to direct, single-nucleotide resolution. In SMM-seq, each genomic DNA fragment is circularized and amplified by rolling-circle amplification, generating long concatemers that contain multiple tandem copies of both original strands. Comparing these copies base-by-base suppresses random polymerase and instrument errors by several orders of magnitude, enabling confident detection of single-nucleotide variants (SNVs) that occur at frequencies far below conventional sequencing noise. We applied SMM-seq to benchmark genotoxic reference compounds. The potent alkylating agent N-ethyl-N-nitrosourea (ENU) produced a steep, dose-dependent rise in mutation burden dominated by A > G, C > T, and A > T substitutions. Potassium bromate ($KBrO_3$), an oxidative stressor, induced mainly C > A transversions consistent with 8-oxo-guanine formation, again in a clear dose-responsive manner. A follow-up panel comprising cisplatin, aristolochic acid, N-nitrosodimethylamine, and benzo[a]pyrene confirmed that SMM-seq reproducibly quantifies both mutation frequencies and distinct spectral fingerprints across diverse mechanisms such as cross-linking, bulky adduct formation, and metabolic activation. By capturing every induced base change rather than only phenotype-altering lesions, SMM-seq corrects the underestimation inherent to reporter-gene assays like the Ames and HPRT tests. Its sensitivity, quantitative accuracy, and ability to deliver mechanistic mutation spectra make it a powerful new approach method for regulatory genotoxicity assessment, high-throughput chemical screening, and the elucidation of DNA-damage signatures in environmental and pharmaceutical safety studies.

Tuesday, 30 June 2026
14:30 - 16:00

Session 4a: Parameterisation of Physiologically-based Pharmacokinetics (PBPK) Modelling for NGRA: Designed to be Protective

Chairs: *Abdulkarim Najjar (Beiersdorf AG) & Clive Roper (Roper Toxicology Consulting Limited)*

O-4a-1

Best practices in physiologically based kinetic (PBK) modelling for next generation risk assessment (NGRA) ABSTRACT #43

Ans Punt[1]
[1]*Unilever SERS*

Bottom-up physiologically based kinetic (PBK) models are developed using in vitro and in silico input data. These models are essential in next-generation risk assessments (NGRA) as they enable the translation of external exposure levels into internal concentrations, which can

then be compared with in vitro bioactivity data. Key assays that are used in bottom-up PBK model development include, for example, in vitro metabolic clearance measurements using human hepatocytes or liver microsomes, plasma protein binding assays, and permeability studies such as Caco-2 for intestine or ex vivo skin penetration studies for dermal absorption. In addition, in silico tools are used to predict partition coefficients based on physicochemical properties like log P and pKa. Although in vitro and in silico data can be effectively used in PBK model development, human clinical data are generally needed for model evaluation. For many substances, such human data are not available, and generating these data is not feasible. Therefore, alternative approaches are needed to establish scientific confidence in bottom-up PBK model predictions. By making use of chemicals with available human clinical data, general insights can be obtained in the performance of bottom-up PBK models. We evaluated, for example, the predictive performance and uncertainty in PBK model-predictions developed with in silico input only or when in vitro input data are used (1, 2). Additionally, a case study on the UV-filter BP-4 was conducted, making decisions based on the chemical characteristics and in vitro transporter kinetic data to include active renal excretion as a key elimination route (3). Overall, the results show the importance of defining best practices to increase confidence in bottom-up PBK model development when no in vivo data are available for validation.

O-4a-2

Strategic in vitro testing for parameterization in PBK model development of cosmetic ingredients
ABSTRACT #59

Nazanin Golbamaki[1]
[1]*L'OREAL*

Background and Objectives Physiologically Based Kinetic (PBK) modelling is crucial for animal-free chemical risk assessment, particularly for cosmetic ingredients. Establishing reliable model parameters requires robust Absorption, Distribution, Metabolism and Elimination (ADME) in vitro and/or in silico data. Guided by OECD Guidance 331 [1], a tiered approach to in vitro testing strategies is presented. PBK models for data-poor cosmetic ingredients are compared to illustrate fit-for-purpose ADME in vitro data, optimizing resources while ensuring a protective approach for regulatory-relevant predictions. Material and Methods The OECD Guidance 331, together with published [2] and newly developed bottom-up PBK models with ADME data in [3], were systematically analysed. The analysis focused on mapping explicit in vitro ADME assay contributions (e.g., skin absorption, permeability, hepatic metabolism, etc.) to PBK model structure complexity. This allowed us to evaluate how specific in vitro data characterize these models for their intended regulatory purpose. Results A clear tiered approach emerged: some chemicals' PBK models were effectively parameterized using only foundational in vitro ADME data or in silico QSARs, allowing for strategic omission of less impactful tests. Other chemicals, requiring complex multi-compartmental PBK models or detailed in vitro to in vivo extrapolations (IVIVE), demanded extensive, specialized in vitro testing. Ultimately, this study allowed for classifying in vitro tests into high utility, context-dependent, and rarely needed categories. Discussion and Conclusion Our findings confirm that a tailored in vitro testing strategy, adaptable to chemical characteristics and model complexity, is vital. It balances data availability and model complexity, enabling accurate prediction of systemic exposures relevant to cosmetic safety. This strategy, tailored to chemical needs, ensured accurate kinetic representation for the case studies. This work creates an initial structure to build upon for verification with larger datasets and a broader chemical space, which strengthens the scientific basis for regulatory acceptance and Next-Generation Risk Assessment.

O-4a-3

Challenges and perspectives for regulatory acceptance of bottom-up NGRA PBK models
ABSTRACT #485

Stephan Schaller[1]
[1]*ESQlabs GmbH*

Bottom-up physiologically based kinetic (PBK) models, parameterised from in vitro and physicochemical data without in vivo calibration, are central to next-generation risk assessment (NGRA). Their regulatory acceptance depends on transparent uncertainty handling, fit-for-purpose evaluation, and demonstrated translation across species, life stages, and endpoints. Three crop protection case studies illustrate the current state of play and open challenges. For boscalid and cinmethylin, a coupled PBK and quantitative systems toxicology model of thyroid hormone homeostasis translates rat-specific in vitro hepatic UGT induction findings to humans, showing that the species-specific rodent effect does not propagate to predicted human thyroid hormone levels. Challenge: defining acceptance criteria for coupled PBK-QST outputs when no human in vivo benchmark exists. For fluopyram, generic avian PBK models for the three regulatory standard species (laying hen, bobwhite quail, mallard), including an ovulation compartment that predicts dietary-to-egg transfer, parameterised from in vitro and physicochemical data, predict species-specific internal exposures for ecotoxicological risk assessment [1]. Challenge: regulatory acceptance of mechanistic predictions in place of default inter-species uncertainty factors. For deltamethrin, a rat-to-human pregnancy PBK model predicts maternal plasma and fetal-brain concentrations at the acceptable daily intake and benchmarks them against the lowest developmental neurotoxicity in vitro testing battery point of departure, in the context of the EFSA IATA on pyrethroid DNT [2,3]. Predicted fetal-brain exposures lie orders of magnitude below the in vitro benchmark, supporting that the current ADI is protective. Challenge: translating in vitro DNT endpoints to internal target-tissue dose with auditable uncertainty. Together, these cases show that bottom-up PBK models, when conservatively parameterised and coupled to mechanistic in vitro endpoints, yield risk assessments that are protective by design. Remaining barriers to broader regulatory adoption are documentation standards, model evaluation guidance, and harmonised uncertainty reporting.

O-4a-4

Quantitative in vitro in vivo extrapolation of nephrotoxicants using PBPK modelling
ABSTRACT #79

Nynke Kramer[1]
[1]*Wageningen University*

Physiologically based pharmacokinetic (PBPK) models are valuable tools in next-generation risk assessment, as they enable the extrapolation of in vitro effective concentrations to in vivo bioequivalent doses—a process known as quantitative in vitro–in vivo extrapolation (QIVIVE). However, most existing PBPK models include only a simplified kidney compartment and assume that glomerular filtration solely drives urinary excretion. As a result, few models can accurately simulate chemical exposures in specific kidney regions, such as the proximal tubule. This limits their ability to predict nephrotoxic doses of chemicals that preferentially distribute to these regions due to active secretion and reabsorption processes. In this presentation, we describe three PBPK models that integrate in vitro kinetic and toxicity data to parameterize and apply PBPK models for estimating human nephrotoxic doses of drugs, pesticides, and environmental contaminants. In the first study, a PBPK model for the chemotherapeutic drug cisplatin was developed for rats and humans. It included a multi-compartment kidney structure and simulated active uptake into the proximal tubule via the organic cation transporter OCT2. This increased the drug's clearance from the bloodstream and its accumulation in the proximal tubule, resulting in simulated plasma concentrations within a 2-fold range of observed in vivo values. Similarly, PBPK models for the pesticides dinoseb and 2,4-dichlorophenoxyacetic acid predicted nephrotoxic doses in rats within a 10-fold range when they incorporated in vitro toxicity and transporter kinetic data from renal proximal tubule epithelial cells transfected with the organic anion transporter OAT1 (RPTEC/TERT1) and conditionally immortalised proximal tubule epithelial cells (ciPTEC). In the final study, a new mechanistic PBPK model for perfluorooctanoic acid (PFOA) was developed and evaluated against human biomonitoring data. The model captures key kinetic processes that drive PFAS distribution in humans, including plasma protein binding,

OATP2B1-mediated enterohepatic circulation, and OAT4-mediated renal reabsorption.

O-4a-5

Evaluation of in vitro methods to investigate long-term hepatic clearance using three low-clearance compounds
ABSTRACT #84

Daniela Lange[1], Abdulkarim Najjar[1], Andreas Schepky[1], Jaqueline Meinhardt[1]
[1]*Beiersdorf AG*

Since the European ban on animal testing for cosmetic ingredients, significant progress has been made in the development and application of New Approach Methodologies (NAMs). While validated NAMs exist for assessing local effects, such as skin and eye irritation, as well as skin sensitization, evaluating the potential for systemic and long-term toxicity requires more complex approaches. A critical factor in determining the maximal systemic concentration of a cosmetic compound is accurately predicting its intrinsic clearance via the liver. However, assessing metabolic stability in the human liver can be challenging, particularly with low-clearance compounds. Short-term assays like suspended primary human hepatocytes (PHH) are used as gold standard to assess hepatic clearance. However, they often fail to capture the complexity and long-term effects of metabolic processes. Their ability to predict in vivo clearance for slowly metabolized compounds is limited since the standard incubation time is only a few hours. Long-term in vitro metabolism assays have gained attention for better replicating the human metabolic system over extended periods. In an ab initio case study to assess the systemic safety of benzyl salicylate in cosmetics [1], we could not confirm hepatic clearance as the primary route for eliminating the main metabolite, Salicylic Acid (SA). In follow-up studies, SA degradation was investigated using long-term metabolism assays. Here we compare clearance and metabolism of three compounds with available in vivo data using PHH, sandwich cultures, co-culture systems with stromal cells, and a tri-culture system that additionally includes endothelial cells. The results show that long-term metabolism models better represent the in vivo metabolism of low-clearance compounds than primary hepatocyte cultures.

Session 4b: Evolution of an OECD Test Guideline and Application Beyond; Integrated NAMs for Skin Sensitisation Hazard and Point of Departure for Risk Assessment

Chairs: *Georgia Reynolds (Unilever R&D) & Bambou Tan (L'Oréal)*

O-4b-1

Transforming skin sensitization: the evolution of the defined approaches for skin sensitization (OECD test guideline 497)
ABSTRACT #46

Emily Reinke[1]
[1]*Inotiv*

Skin sensitization has been an area of significant advancement in the area of new approach methodologies (NAM) and the field has been leading in validation approaches and regulatory acceptance of NAMs. Development of these NAMs has centered around an understanding of the mechanisms of skin sensitization and the key events published in the adverse outcome pathway. Key to the advancement of these approaches has been the Organisation for Economic Co-operation and Development (OECD) test guideline program. Development and validation of individual NAMs has resulted in key event-based OECD Test Guidelines (TG) (TG 442C, TG 442D, and TG 442E) for developed in vitro methodologies. Recognition that these methods as stand-alone approaches were not adequate to make a decision on skin sensitization potential led to the development of multiple defined approaches. A subset of these DAs were evaluated for inclusion in a new OECD TG. DAs allow integration of data from the TG 442 assays and established in silico prediction models in a structured manner and have a defined data interpretation procedure to remove the need for expert judgement on a decision. The adoption of OECD TG 497 in 2021 included DAs for hazard identification and sub-potency categorization. Since this time, numerous advancements have been made, and new projects to the OECD to expand the

scope of OECD TG 497. These projects include the addition of similar methods from the TG 442 series and the inclusion of a new DA for deriving a point of departure. This presentation will detail the performance and evaluation of the new methods in the DAs and how they are intended to be applied.

O-4b-2

Best practice guidance to advance the acceptance of skin sensitization NAMs and NGRA
ABSTRACT #87

Donna Macmillan[1]
[1]*International Collaboration on Cosmetics Safety*

Skin sensitization has long served as an example for how a toxicological endpoint can transition from traditional in vivo testing to non-animal approaches known as New Approach Methodologies (NAMs). Skin sensitization NAMs (in chemico and in vitro methods, in silico models) have been used to develop Defined Approaches (DAs) which provide qualitative or quantitative predictions and several DAs have been formalized in OECD Guideline 497. While the scientific maturity of NAMs for skin sensitization is well established, uptake for regulatory decision-making remains variable. Scientific consensus building and alignment across stakeholders continues to be a barrier to broader implementation. To address this, the International Collaboration on Cosmetics Safety (ICCS) initiated a series of Best Practice Guidance (BPG) documents, with skin sensitization selected as the starting point given the field's maturity and level of regulatory acceptability. The BPG for skin sensitization distils current scientific understanding and practical experience into a structured resource to guide the generation, evaluation, and interpretation of NAM data. It reflects the collaborative input from multidisciplinary experts within ICCS and aims to support regulatory acceptance by promoting reproducibility, transparency, and consistency in data interpretation. By aligning technical best practices with evolving regulatory expectations, the BPG provides a scientifically grounded framework for the application of NAMs in both hazard and safety-based contexts. This presentation will explore the structure and scientific underpinnings of the skin sensitization BPG, its practical applications, and the methodological and collaborative insights gained throughout its development.

O-4b-3

SARA-ICE DA: defining a PoD for skin sensitisation risk assessment
ABSTRACT #39

Georgia Reynolds[1]
[1]*Unilever*

To advance beyond hazard and sub-potency categorisation, it is essential to adopt approaches that leverage new approach methods (NAMs) for skin sensitisation to define points of departure (PoDs). NAM-derived PoDs enable quantitative safety assessments without relying on animal testing. Several published methods support PoD derivation from NAM data, including the Bayesian statistical model SARA-ICE DA—the first of its kind accepted into OECD TG 497. SARA-ICE DA estimates an ED01, the dose associated with a 1% chance of human skin sensitisation in a human predictive patch test (HPPT). The PoD is defined as the mean ED01, assuming the substance is a sensitiser. The model accepts diverse input data (in chemico, in vitro, in vivo) and does not require a fixed dataset, allowing flexible, iterative use based on available data. The SARA-ICE PoD, derived from NAM inputs, was compared to reference values including the Local Lymph Node Assay (LLNA) median-like location parameter (MLLP) and the dose per skin area that sensitises one subject (DSA1+). Across comparisons, the absolute bias between SARA-ICE DA PoDs and reference values was approximately 2-fold. Predictive performance against DSA1+ was comparable to that of LLNA MLLP. A web application has recently been developed for local installation, making SARA-ICE DA accessible to all stakeholders. To demonstrate application, the presentation will showcase case studies applying SARA-ICE DA to materials with varying NAM data. Example exposures will be applied as part of the case studies and a method applied to demonstrate how acceptable margins of exposure can be derived and applied within existing safety assessment frameworks. This presentation will emphasise the key features, evaluation, and practical application of the SARA-ICE DA.

Panel discussion on best practise in applying NAMs in this manner will be encouraged at the end of the talk to conclude the session.

O-4b-4

Integrated approaches for skin sensitization assessment: optimizing predictive approaches within best guidance practices
ABSTRACT #130

Nathalie Alépée[1], Fleur Tourneix[1]
[1]*L'Oreal R&I*

Skin sensitization remains a persistent public health challenge, demanding robust assessments to protect human health. In response to this challenge, the emergence of New Approach Methodologies (NAMs) is revolutionizing risk assessment, offering ethical and efficient alternatives to traditional animal testing. OECD Test Guideline 497 establishes a crucial regulatory framework for in vitro skin sensitization, emphasizing the judicious integration of diverse information. For successful implementation of these NAMs, adherence to best practice guidance, such as that published by the ICCS, is paramount; it guides data interpretation and integration towards reliable hazard characterization. During this presentation, we will delve into the practical application of advanced predictive tools within this established framework. We will particularly highlight the SARA ICE model, a key module within OECD TG 497, underscoring the crucial importance of thoughtful engagement with its input parameters. We will explore how modulating these parameters – related to chemical structure, metabolic activation, or existing in vitro/in chemico data (KE1, KE2, KE3) – can significantly refine hazard characterization. In parallel, we will discuss the evaluation of other predictive approaches, such as the Bayesian Skin-BN model developed recently. These complementary approaches, leveraging similar input parameters (from KE1, KE2, KE3 assays and physicochemical properties), offer alternative perspectives and strengthen the overall weight-of-evidence for skin sensitization assessment, paving the way for a more comprehensive and reliable risk assessment strategy.

Tuesday, 30 June 2026
16:30 - 18:30

Session 5: From Research to Regulation: standardising innovative non-animal technologies

Chairs: *Julia M. Malinowska (European Commission, Joint Research Centre) & Iva Sovadinova (RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic)*

O-5-1

The role of standards in the development and application of iPSC-derived assays and organoids
ABSTRACT #30

David Pamies[1]
[1]*University of Lausanne.*

Human induced pluripotent stem cells (hiPSCs) have transformed in vitro research by providing a renewable, genetically diverse, and physiologically relevant source of human cells. These advances are driving the development of complex human-based models, also known as microphysiological systems (MPSs), such as organoids, which hold great potential for disease modeling, toxicology, drug screening, and personalized medicine. However, this promise is often limited by the lack of protocol harmonization, variability between iPSC lines, and challenges related to differentiation control and reproducibility. To fully realize the potential of hiPSC-derived assays and organoid-based models, robust quality control and international standardization are essential. Initiatives such as Good Cell Culture Practice (GCCP 2.0), OECD guidelines, the ISSCR Standards, and collaborative efforts by networks like ISCBI and EBiSC are paving the way toward improved reproducibility, regulatory acceptance, and cross-laboratory validation. This presentation will explore the evolving landscape of standardization for iPSC-based in vitro models, with a focus on organoids and other MPSs. Key regulatory milestones, validated assay examples, and proposed quality management strategies will be discussed to strengthen confidence in the use of these innovative systems.

O-5-2

Qualification of a human 3D liver-on-chip model: establishing a cross-pharma trial to evaluate ADME and toxicity predictions in pre-clinical development
ABSTRACT #24

André Lopes Rodrigues[1], Clara Ramon Lozano[2], Katharina Schimek[34], Thi Phuong Tao[34], Katarina Breitholtz[5], Dorra El Hajjam[6], Johanna Troberg[7], Melina Malinen[7], Chloe Korlowski[8], Anaëlle Pay[9], Lucile Rabiet[8], Grégoire Harichaux[9], Alexandre Martins[10], Catarina Chaves[10], Sebastian Prill[11], Behnam Amiri[12], Jure Fabjan[12], Christian Maass[12], Shweta Bendre[13], Darshana Amberkar[13], Mario Beilmann[13], Rhiannon David[11], Teija Oinonen[7], Amelie Moreau[2], Liisa Vilén[5], Reyk Horland[34], Reiner Class[1], Benoit Cox[1]
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[9]*BA & NCPK, DMPK, Translational Medicine Department, Servier, Gif-sur-Yvette, France*
[10]*Laboratory Sciences, Translational Medicine Unit, Research and Development, Sanofi*
[11]*Safety Innovation, Safety Sciences, Clinical Pharmacology & Safety Sciences, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden and Cambridge, UK*
[12]*MPSlabs, ESQlabs GmbH, Saterland, Germany*
[13]*Global Nonclinical Safety & DMPK, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany*

Safety concerns, including drug-induced liver injury (DILI), are one of the primary reasons that lead to the discontinuation of drug candidates during clinical development, highlighting the need for better preclinical testing methods. Traditional animal models have limited relevance to humans, prompting the development of complex in vitro models (CIVM). In this context, we have established a pathway to validate a 3D liver-CIVM according to the guidelines outlined by the Innovation and Quality (IQ) consortium [1]. This study describes a Liver Ring Trial (LRT) as a joint effort by 6 international pharmaceutical companies, a platform developer, and a computational expert company to test the overall reproducibility of a liver microphysiological system (MPS) across different laboratories. This qualification strategy starts by evaluating the biological functionality and reproducibility of the liver-CIVM, which is context-of-use agnostic. Hepatic biomarkers such as albumin, urea, alanine and aspartate transaminase (ALT, AST) are measured, alongside an ATP measurement for cellular viability. In a second stage, 9 reference drugs were selected to validate the reproducibility of results in two different contexts of use: i) DILI and ii) intrinsic clearance. Parent drug concentrations are monitored throughout the incubation to enable drug clearance estimations, as well as to link the drug concentration to any potential toxic effect. Recognizing the challenge posed by interlaboratory variability, we have devised a Standard Operating Procedure (SOP) aligned with regulatory standards, which randomly and blindly attributes test items to each member for the qualification rounds. By addressing interlaboratory variability and delivering a comprehensive qualification of the 3D MPS liver model, our study contributes significantly to establishing MPS as a standard screening tool in pharmaceutical drug development. This rigorous qualification process is pivotal in ensuring the dependability and reproducibility of results across diverse laboratories, ultimately propelling the field toward more precise and cost-effective preclinical assessments.

O-5-3

Development and validation of a MAT-assay-variant based on assay-ready THP-1 monocyte and a sensitive qPCR read-out
ABSTRACT #51

Felix Hackstein[1], Marvin Lange[2], Lukas Focke[1], Oliver Wehmeier[1]
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[2]*HAW Hamburg, Faculty of Life Science, Hamburg, Germany*

Microbial, viral and chemical residuals can trigger fever in humans. Therefore, all pharmaceuticals and medical devices which get into a patient's body need to be tested to be free of any of these pyrogens. For a long time, accepted in vitro assays could only detect a subset of pyrogens or were not sensitive enough. So, the animal-based Rabbit Pyrogen Test (RPT) remained the gold standard. Just in July 2025 the European Pharmacopoeia fully abandoned the RPT to support New Approach Methodologies like the Monocyte Activation Test (MAT). This in vitro assay is based on the release of inflammatory cytokines like TNF-a, IL-1β and IL-6 from human monocytes, commonly detected by ELISA to quantify the pyrogenic response. Here we present the development and validation of a MAT-assay variant built on primed assay-ready THP-1 monocytes and quantitative PCR (qPCR) to measure the pyrogenic markers already at the level of gene expression. The assay detects all tested endotoxin and non-endotoxin reference pyrogens. Validation data for robustness, assay precision, accuracy, linearity, and sensitivity will be shown. By using assay ready cells, the qPCR read-out, and the improved protocol, a robust assay could be developed which delivers precise data already one day earlier than with the common protocol, while the sensitivity of the assay could be even slightly increased.

O-5-4

Implementing high throughput testing to accelerate the adoption of in vitro methods
ABSTRACT #40

Donatella Carpi[1], Aurora Fassi[1], Roman Liska[1], Peter Macko[1], Julia Malinowska[1], Emilio Mendoza[1], Stavroula Sampani[1], Taina Palosaari[1], Maurice Whelan[1]
[1]*European Commission, Joint Research Centre (JRC), Ispra, Italy*

The development of international test guidelines for use under the Mutual Acceptance of Data of the Organisation for Economic Co-operation and Development requires rigorous validation of newly proposed test methods. In this context, validation is the process of assessing the reliability and relevance of a method intended for a particular purpose, such as generating information on adverse toxicological effects of regulatory concern. Assessing the relevance of in vitro methods involves evaluating multiple parameters including sensitivity, specificity, concentration-response relationships, and the applicability domain with respect to the chemical space covered. Traditionally, generating the large datasets needed for these evaluations has relied on manual testing in one or more laboratories, a process that is time-consuming and resource intensive. High Throughput Testing (HTT) leverages the use of advanced automated technologies, such as liquid handlers and robotic arms, to enable faster and more consistent data generation. Furthermore, integrating additional information such as in vitro kinetics can facilitate the interpretation of in vitro toxicity data. However, successful HTT requires careful translation of manual protocols into suitably designed automated processes. This presentation will illustrate key considerations and practical steps for automating in vitro methods to support their validation, using practical examples of studies conducted on the HTT platforms of the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). The Open ECVAM initiative will also be described that provides open access to our HTT laboratory to external users interested in the generation of large, high-quality datasets from toxicologically relevant chemical libraries. Embracing the aims of the EU Life Science Strategy, our Open ECVAM programme aims to enhance knowledge sharing, promote standardisation as well as support regulatory uptake and commercialisation of innovative in vitro approaches for safety assessment of chemicals.

O-5-5

Barcoding cell toxicity - using the cell painting plus method for phenotypic profiling and detailed MOA prediction ABSTRACT #9

Elena von Coburg[1], Marlene Wedler[12], Jose M. Muino[13], Floriane Odje[4], Christopher Wolff[5], Nils Körber[6], Jens von Kries[5], Andrea Volkamer[4], Sebastian Dunst[1], Shu Liu[1]
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[5]*Screening Unit, Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Berlin, Germany*
[6]*Centre for Artificial Intelligence in Public Health Research, Robert Koch Institute, Berlin, Germany*

To protect human health, EU and national legislations request safety testing of industrial/agricultural chemicals and pharmaceuticals prior to their marketing. This includes identification of substances with endocrine activities that can cause carcinogenicity or reproduction toxicity. In that context, expanding the 'New Approach Methodology' (NAM) toolbox with imaging-based, human-relevant cell models and computational approaches is becoming increasingly important to bridge the gap between complex in vivo methods (detecting adverse health effects) and simplified in chemico/in vitro methods (resolving endocrine MoA) [1]. We use Cell Painting-based high-throughput phenotypic profiling in combination with AI-driven approaches for extracting more detailed morphological and functional information from single cells [2] to improve MoA characterization in regulatory toxicology. Our novel Cell Painting PLUS (CPP) method [3] captures substance-induced phenotypic changes in cell morphology and organelle structure ('cell pathologies'), which represent an early indication of tissue and organ dysfunction that can ultimately lead to adverse health effects. The cell organelles are labeled with fluorescent dyes in an iterative-staining elution process, imaged with automated HCS microscopes, and analyzed with AI-based image and data analysis software. In this way, the phenotypic responses of cells to substance exposure are converted into characteristic 'barcode-like' activity profiles ('morphological fingerprints') that include hundreds of cellular readouts. We currently combine the CPP method with endocrine-specific prediction models into a defined test procedure (PaintED Assay) for detection and differentiation of endocrine activities (ER, PR, GR). That includes the i) standardization and automation of experimental and analytical routines, ii) evaluation of technical transferability and reliability, iii) definition of robust prediction models including quality control and acceptance criteria, and iv) establishment of a 'validation-ready' SOP. The overall goal is to establish the PaintED Assay as a new animal-free, human-relevant NAM for endocrine endpoints in safety assessment of industrial/agricultural chemicals and pharmaceuticals.

O-5-6

Standardising omics data for regulatory toxicology: frameworks, progress, and the path forward ABSTRACT #21

Florian Caiment[1]
[1]*Maastricht University*

In the transition toward innovative non-animal technologies, omics-driven evidence offers an unparalleled lens on the molecular cascades triggered by chemical exposure. Yet transforming these rich datasets into actionable, regulator-ready knowledge remains challenging. This talk will survey the current regulatory landscape for omics data, spotlighting both achievements and hurdles. We first examine the OECD Omics Reporting Framework (OORF (1)) and Metabolomics Reporting Framework (MRF), which translate complex raw outputs into FAIR-compliant packages that facilitate transparent peer review and regulatory appraisal. Building on these foundations, the R-ODAF pipeline (Omics Data Analysis Framework for regulatory applications

(2)) will be introduced as a robust analytical scaffold that filters raw reads, applies stringent statistical thresholds, and generates reproducible lists of differentially expressed genes, thereby reducing false-positive rates and bolstering data credibility. Moving from analysis to interpretation, the ECETOC taskforce on Good Practices in Omics Data Interpretation for Regulatory will be showcased as a pragmatic template for quantitative hazard identification suitable for regulatory decisions. Finally, the talk will appraise the degree of uptake of these frameworks by regulatory authorities, identifying persistent gaps, and discuss the latest development in this field. Concrete recommendations will be proposed to accelerate formal validation and routine regulatory use, positioning omics as a cornerstone of next-generation, animal-free safety assessment. By charting the trajectory from raw data to regulatory decision-making, this presentation underscores how rigorous standardization can unlock the full potential of omics technologies and expedite their acceptance alongside other innovative non-animal methodologies.

O-5-7

Accelerating regulatory acceptance through international cross-site ring trials: current developments in drug development and food safety
ABSTRACT #11

Matthias Gossmann[1]
[1]*innoVitro GmbH*

The journey from laboratory research to regulatory acceptance requires a broad spectrum of stakeholders—including technology developers, end-users, and regulatory authorities—to gain confidence in non-animal technologies (NATs). While each group brings distinct expectations and evaluative criteria to the table, public-private partnerships have emerged as powerful enablers in advancing this transition. A prominent example is the Comprehensive In Vitro Proarrhythmia Assay (CiPA) initiative, which extensively explored the utility of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) as in vitro models for assessing proarrhythmic risk. Notably, this was the first large-scale effort to evaluate hiPSC-CMs as potential replacements for animal-based models in the mandated assessment of hERG channel and other drug-induced arrhythmic liabilities. Building upon this foundation, more recent investigations under the same framework have expanded the scope of hiPSC-based new approach methodologies (NAMs). These studies examined their suitability for chronic cardiotoxicity testing and for evaluating the safety of complex mixtures such as botanical extracts. In the CardioTox study, led by HESI, 23 test sites from both industry and academia used 10 different assay platforms to evaluate 12 blinded compounds with known chronic cardiotoxic profiles, including doxorubicin, pentamidine, and sunitinib. Additionally, an initiative by the FDA and NIEHS assessed the cardiac effects and potential cardiotoxicity of 16 botanical extracts. This work presents key findings from both initiatives, highlighting the general applicability of hiPSC-CM–based NAMs, their current limitations, and the future directions necessary to further validate and integrate these models into regulatory frameworks.

Wednesday, 1 July 2026
09:00 - 10:30

Session 6a: Advancing the Use of NAMs in Food Safety

Chairs: *Ricard Celorio (FoodDrinkEurope) & Iva Sovadinova (RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic)*

O-6a-1

Systemic effects evaluation using NAM toolboxes: key lessons learned from real-world cases
ABSTRACT #122

Philip Marx-Stoelting[1]
[1]*German Federal Instiute for Risk Assessment*

Several EU projects have been working on the development of new approach methodologies (NAM) resulting in an increasing number of in vitro and in silico for the testing of potential toxic effects of chemical substances. Despite the variety of methods available regulatory uptake

remains a challenge. This is partially due to limited readiness of methods not sufficiently adressing the level of robustness or validity needed for regulatory implementation. On the other hand, at the regulatory level a broad understanding of the chances of NAM with respect to address human relevance, analyse effects of multiple compounds and mixtures by screening approaches or contribute to a better understanding of mechanisms of toxicity needs to be created. Projects like PARC or the ASPIS cluster projects involving method developers as well as regulators and ensuring a high level of exchange and creation of a common understanding of needs and challenges will help to overcome these difficulties. This has been particularly undertaken by assembling several methods to test batteries, evaluating a complex endpoint like systemic toxicity. Examples from these projects will be shown to demonstrate strength but also remining challenges. This presentation describes PARC and other current European research and regulatory initiatives ensuring the implementation of 21st century toxicology methods in the 21st century

O-6a-2

The role of NAMs in transforming to human based safety assessment
ABSTRACT #124

Cyrille Krul[1], Anne Kienhuis[23], Juliette Legler[2], Bas Blaauboer[2], Geraldine Borja[4]
[1]*University of Applied Sciences Utrecht*
[2]*Utrecht University*
[3]*RIVM*
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There is increasing political and societal pressure to reduce animal testing, supported by European consumers and regulatory initiatives such as the European Roadmap Towards Phasing Out Animal Testing for Chemical Safety Assessments and EFSA's 2022 Roadmap for Action on NAMs. For the food and beverage sector, New Approach Methodologies (NAMs) offer faster, more cost-effective, and human-relevant methods that align better with modern risk assessment principles and ethical standards. The Virtual Human Platform for Safety Assessment (VHP4Safety) project aims to protect human health and improve safety assessment without the use of animals. This interoperable platform offers in silico tools and integrate human-relevant data, including in vitro assays representing key events in Adverse Outcome Pathway (AOP) networks. Artificial intelligence supports data integration and predictive modelling of chemical safety. Case studies within VHP4Safety are designed to reflect human-relevant exposure scenarios, including vulnerable populations, often overlooked in animal studies. A dedicated food safety case study—developed in collaboration with academia, industry, regulators, and societal stakeholders—could demonstrate the applicability of NAMs and address uncertainties associated with novel approaches. Validation using real-life human data is essential to assess scientific robustness and regulatory relevance. The active involvement of diverse stakeholders fosters responsible and transparent data sharing, which is crucial for building trust in NAMs and facilitating regulatory acceptance. This interdisciplinary collaboration—combining toxicology, data science, and social science—is key to establishing new platforms such as VHP4Safety, as a credible and accepted framework for future safety assessments.

O-6a-3

Integrating NAMs to tood safety: case studies advancing risk assessment and mechanistic understanding
ABSTRACT #93

Elena Reale[1], Danilo Basili[1], Bennard van Ravenzwaay[2], Styliani Fragki[3], Hennike Kamp[4], Angela Mally[5], Alicia Paini[3], Stephan Schaller[3], Marco Siccardi[3], Marjorie Guitard Uldry[1], Alexandre Panchaud[1], Gina Montoya[1]
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New Approach Methodologies (NAMs) are central to advancing next-generation risk assessment (NGRA) in food safety, offering refined, mechanistically informed alternatives to traditional animal testing. NGRA relies on a tiered strategy: Tier 0 involves problem

formulation and data gathering, Tier 1 applies in silico methods, high-throughput screening and systemic testing, and Tier 2 integrates targeted NAMs to refine assessments. Here, we present case studies illustrating the application of this approach to food-relevant molecules, including bioactives and contaminants. For the isoflavones genistein and daidzein, in depth analysis of existing animal plasma metabolomics data revealed differences in their mechanisms of toxicity, including divergent NOAELs for estrogenic effects. These insights highlight the value of NAMs in differentiating hazard profiles among structurally related food compounds, particularly for informed cumulative risk assessment. For atractyligenin derivatives, known to impair mitochondrial respiration, we combined in vitro bioassays with physiologically based kinetic (PBK) modeling to perform quantitative in vitro-to-in vivo extrapolation (qIVIVE). This enabled derivation of preliminary human-relevant exposure limits to support safety evaluations in food and consumer products. For 2-chloroethanol (2-CE), a contaminant currently assessed as toxicologically equivalent to ethylene oxide due to data gaps, we are generating in silico and in vitro data to fill this void and inform risk assessment for 2-CE. Our hypothesis-driven approach explores whether DNA damage occurs only at exposure levels that saturate glutathione (GSH)-dependent detoxification of the reactive metabolite 2-chloroacetaldehyde. For alkylfurans, assumed to exert additive toxicity with furan due to structural similarity, we applied in silico tools to predict their metabolites and genotoxicity. We combined predictions with in vitro biotransformation and bioactivity assays to compare the effects of alkylfurans and their metabolites to furan. Together, these case studies demonstrate how NAMs support evidence-based decision-making, improve mechanistic understanding, and build confidence in non-animal approaches within the evolving landscape of food safety assessment.

O-6a-4

Using NAMs in a weight of evidence approach
ABSTRACT #102

Franck Atienzar[1]

[1]*The Coca Cola Company*

The use of NAMs (New Approach Methods) into food safety assessments is limited because many NAMs lack exhaustive scientific validations and regulatory acceptance. For instance, EFSA recently stipulated that “When these methodologies are qualified or become validated as alternative approaches, applicants are encouraged to make use of them to provide data on the safety of the novel food” (EFSA, 2024). Unfortunately, the full validation of NAMs can be extremely time-consuming and may take a decade particularly when comprehensive interlaboratory validation trials are performed based on OECD guidelines. Nevertheless, NAMs, not undergoing an official validation process, can still play a role in hazard and risk assessment exercises particularly when they are not analyzed in isolation but in a Weight of Evidence Approach (WoE). Examples of NAMs will be provided based on real cases from the pharmaceutical and cosmetic industry. For instance, in the cosmetic field, the use of a genotoxicity reporter assay combined with a 3D-reconstructed skin model allowed to derisk genotoxicity obtained in an in vitro mammalian assay. In a pharmaceutical project, two families of chemicals developed to cure Alzheimer’s disease were associated with a very high rate of genotoxicity according to the Human Greenscreen and micronucleus assay in TK6 cells. The supplementation of these two in vitro tests with iron allowed to (i) derisk the family of chemicals, (ii) refine the mechanism of action leading to genotoxicity and (iii) move the project forward. Examples from the literature will be also described. For example, recently, Brorby et al. (2025) stressed that NAMs could provide mechanistic understanding and allow to perform risk assessment of complex mixtures such as smoked flavors in combination to exposure assessment. In conclusion, all these examples illustrate the usefulness of NAMs not only to provide mechanistic information but also to perform risk assessment using a WoE approach.

O-6a-5

Application of new approach methodologies (NAMs) in the risk assessment of food/feed contaminants and botanicals

ABSTRACT #442

Eirini Kouloura[1], Edoardo Carnesecchi[1], Andrea Gissi[1], Alexis Nathanail[1], Chantra Eskes[1]
[1]*European Food Safety Authority (EFSA), Italy*

New Approach Methodologies (NAMs) are increasingly applied in chemical risk assessment to support a more mechanistic, efficient and animal-free evaluation of hazards. EFSA has progressively integrated NAMs into scientific assessments across food safety domains. These include in silico approaches such as quantitative structure–activity relationship (QSAR) models as well as artificial intelligence (AI)-based tools. This presentation provides an overview of EFSA's current and evolving use of NAMs in the risk assessment of contaminants, with a focus on genotoxicity assessment. The risk assessment of contaminants is primarily based on evaluation of existing evidence, including experimental studies reported in peer-reviewed literature. In this context, computational and AI-based approaches may be applied as screening tools to efficiently identify potential hazards and support the evaluation process. In silico methods including (Q)SAR models and read-across approaches, are used to support hazard identification and characterisation, prioritise substances for further investigation, and address data gaps where experimental evidence is limited. These approaches are applied within a transparent and structured framework, in line with EFSA's guidance documents for the evaluation of reliability, relevance and uncertainty. A case study will also be presented on the application of (Q)SAR methodologies within EFSA's Compendium of Botanicals database. Given the large number of botanicals used as food or feed and the frequent lack of substance-specific toxicity data, EFSA has implemented a systematic in silico screening strategy. High-quality (Q)SAR platforms were used to generate predictions for several toxicological endpoints, for substances present in botanicals identified as being of potential concern. Where available, experimental data are given greater weight in an assessment, while in silico predictions are used as supporting information to inform the safety assessment of botanicals. Overall, EFSA's experience demonstrates how NAMs can enhance scientific robustness, improve efficiency and support the transition towards next-generation risk assessment in food and feed risk assessment.

Session 6b: Replacing the use of animals in therapeutic monoclonal antibody drug approvals

Chairs: *Jeffrey Brown (PETA Science Consortium International e.V.) & Andreas Stucki (PETA Science Consortium International e.V.)*

O-6b-1

The current landscape of non-animal preclinical assessment for monoclonal antibodies and biopharmaceuticals

ABSTRACT #104

Leander Grode[1]
[1]*Serum Life Science Europe GmbH*

The development of biopharmaceuticals is a complex process, traditionally involving extensive use of animal models to assess safety and efficacy. Advances in scientific methodologies and ethical considerations are now driving a shift towards non-animal preclinical assessment techniques. This shift is guided by the 3R principle: Replacement (substituting animal tests with alternative methods), Reduction (minimizing the number of animals used), and Refinement (enhancing techniques to reduce animal suffering). Key non-animal methods include in vitro techniques and computational models. Cell-based assays and organ-on-a-chip systems allow for detailed assessment of drug interactions and mimic human organ functions. Computational models help predict biological responses and potential toxicity. Regulatory acceptance is crucial for adopting these methodologies. Engaging with regulatory agencies through scientific advice meetings ensures alignment with expectations and supports the rationale for non-animal methods. This overview will provide insights into the latest scientific advancements, regulatory frameworks, and practical considerations for implementing non-animal methodologies, ultimately aiming to improve clinical outcomes.

O-6b-2

Approaching the development and regulatory review of therapeutic monoclonal antibodies without using animal studies
ABSTRACT #100

Jeffrey Brown[1], Jessica Perrin[1]
[1]*PETA Science Consortium International e.V.*

Many new drugs—including therapeutic monoclonal antibodies and similar classes—are more individualized and human-specific than ever before, and developers and regulatory bodies are agreeing that drugs such as these cannot always be adequately tested with animal models. Notably, several sponsors have published their experience gaining regulatory approval to start first-in-human clinical trials exclusively on the basis of in vitro studies. Even more developers have published examples of using in vitro data to support safety or dose selection prior to human tests without animal data alongside it. The current preclinical testing standard, however, usually means exhausting all possible animal-based methods before considering non-animal techniques. This session will be organized around the theme of "when and how to use emerging technologies rather than defaulting to animal models for pharmaceutical safety testing during monoclonal antibody drug development and approval." One speaker has been providing expert consultant services in the biopharmaceutical space for over 15 years and will share his perspective on the current state of non-animal methods in new drug preclinical assessment. A second speaker is the co-founder of a company offering recombinant antibody development services. She will discuss her recent experience developing a therapeutic monoclonal antibody candidate drug without using animals. As the final speaker, I will talk about my organization's experience bringing a non-animal preclinical safety weight-of-evidence approach for regulatory review (including by the US Food and Drug Administration (FDA), the Paul-Ehrlich-Institut, and the European Medicines Agency), as the strategy applies to the organization's sponsored candidate monoclonal antibody therapeutic. This is all against a backdrop of a rapidly developing policy under which the FDA will prioritize ending the use of animals in therapeutic mAb testing within the coming three to five years.

O-6b-3

Beyond the saddle: fully human antibodies against diphtheria toxin from animal-free discovery platforms
ABSTRACT #96

Esther V. Wenzel[1]
[1]*Abcalis GmbH*

Modern diphtheria therapy still relies on equine-derived antitoxin (DAT), whose production involves animal suffering, batch-to-batch variability, and risk of adverse immune reactions in patients. Our study demonstrates a fully animal-free, phage display–based pipeline to discover human monoclonal antibodies as a potential replacement for DAT in treating diphtheria toxin exposure. We constructed two immune libraries using peripheral blood mononuclear cells from vaccinated human donors and employed complementary phage display panning strategies to select specific single-chain variable fragments (scFvs) against diphtheria toxin. Hundreds of unique antibody fragments were isolated, a substantial number of which showed potent neutralizing activity in vitro. A subset of these were further developed into full-length human IgG1 antibodies for functional and biophysical characterization. These recombinant antibodies showed strong neutralizing capabilities in cell-based assays and demonstrated target engagement across different functional domains of the diphtheria toxin. Importantly, combinations of selected monoclonal antibodies outperformed single agents in neutralization potency, both in vitro and in an in vivo intradermal challenge assay. This synergy suggests a viable therapeutic alternative to polyclonal equine serum, with improved consistency and safety. Our findings highlight the potential of phage display and human donor-derived libraries as powerful, ethical alternatives to traditional animal-based antibody generation. This approach not only avoids animal immunization entirely but also yields therapeutics with high specificity, potency, and scalability. By advancing these fully human monoclonal antibodies toward clinical use, we provide a compelling proof-of-concept for replacing animals in the development and regulatory approval of antitoxin biologics.

Wednesday, 1 July 2026
11:00 - 13:00

Session 7a: Complex chemical mixtures across the environment-food-human continuum

Chairs: *Anne Marie Vinggaard (Technical University of Denmark) & Mohamed Fathi Abdallah (Department of Food Science, Aarhus University, Denmark.)*

O-7a-1

Effects of chemical mixtures across the continuum from the environment to humans
ABSTRACT #60

Beate Escher[1], Jean-Philippe Antignac[2], Marc Audebert[3], Peter Cenjin[4], Timo Hamers[4], Maria Joao Valente[5], Laure Khoury[3], Maria König[6], Marja Lamoree[4], Jungeun Lee[7], Solene Motteau[2], Yanying Ma[5], Maria Margalef[4], Kostja Renko[8], Martin Scholze[9], Anne Marie Vinggaard[5]
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[2]*Laberca, Oniris*
[3]*Preditox*
[4]*Vrije Universiteit Amsterdam*
[5]*Technical University of Denmark*
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[8]*German Federal Institute for Risk Assessment BfR*
[9]*Brunel University*

One objective of the PANORAMIX project was to assess the combined effects of complex real-life chemical mixtures across the environment-food-human continuum. Key exposure sources included influent and effluent from wastewater treatment plants, representing anthropogenic emissions, surface water with fish as aquatic biota and food source, and cow milk (conventional and organic) as a terrestrial and dietary exposure proxy. Additionally, pooled human biological samples—breast milk, adult serum, and umbilical cord serum—were analyzed to reflect average European internal human chemical exposure. Initial efforts involved extraction and chemical analysis of these pooled samples, encompassing both endogenous compounds and environmental pollutants. A test battery of 22 in vitro bioassays was developed, targeting key toxicity domains such as developmental neurotoxicity, endocrine disruption (thyroid and reproductive hormones), genotoxicity, and adaptive stress responses. Whole mixture testing of all extracts was performed and activity was recorded for most endpoints, except for genotoxicty, and except for drinking or bottled water. From this battery, six robust assays were selected for component-based mixture testing of 24 chemicals that were present in at least 3 water/food/human samples and quantified. Mixtures were composed of the chemicals in ratios mimicking their occurrence in 14 environmental and biological samples (excluding drinking and bottled water). These mixtures were tested across the selected assays, revealing that all, except for genotoxicity, exhibited biological activity. Commonly observed effects included cytotoxicity, thyroid hormone disruption (via transthyretin binding and NIS activity), anti-androgenic activity, oxidative stress, and neurotoxicity. Mixture modeling showed that observed effects were largely predictable using concentration addition models and that 'something from nothing' was observed in several cases. However, these models only accounted for a portion of the observed activity in the original pooled extracts, indicating the influence of unidentified chemicals or matrix effects, underscoring the need for future methodologies to differentiate between endogenous and pollutant-induced effects.

O-7a-2

Chemical mixtures in human cord blood: bioassay responses and links to early childhood health effects
ABSTRACT #63

Maria Margalef[1], Erich Batzella[2], Martin Scholze[3], Beate I. Escher[45], Maria João Valente[6], Maria König[4], Henriette Boye[7], Tina Kold Jensen[2], Anne Marie Vinggaard[6], Timo Hamers[1], Marja Lamoree[1]
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[5]*Environmental Toxicology, Department of Geoscience, Eberhard Karls University Tübingen*
[6]*National Food Institute, Technical University of Denmark*
[7]*Odense University Hospital, Region of Southern Denmark*

The development of the nervous and reproductive systems during early life stages is critical for achieving optimal health and functioning throughout adulthood. These systems are particularly vulnerable to disruption by environmental exposures during fetal and early postnatal development, with potential long-term consequences for cognitive function, behavior, fertility, and reproductive health. Moreover, exposure to multiple chemicals could impact the risks associated with individual substances. The EU funded Panoramix project is the first to apply interdisciplinary strategies to assess the effect of complex chemical mixtures extracted from human cord blood serum. This study analyzed a subset of 750 children from the Odense Child Cohort (OCC), for whom data were available on intelligence quotient (IQ), language development, attention-deficit/hyperactivity disorder (ADHD), and anogenital distance (AGD), a marker for prenatal androgen action. Cord blood extracts from individual samples were evaluated using a comprehensive battery of in vitro bioassays targeting molecular initiating events and key events across key adverse outcome pathways for these endpoints. These included assays indicative of neurotoxicity, thyroid hormone system disruption, genotoxicity, and anti-androgenic activity, offering mechanistic insights into how prenatal exposure to complex chemical mixtures may affect critical developmental processes. Advanced computational approaches were used to study associations between bioassay responses in cord blood and maternal chemical mixtures with the health outcomes. In addition, effect-directed analysis was used to identify the chemical drivers behind observed biological effects in vitro. This study underscores the importance of assessing prenatal exposure to chemical mixtures rather than individual chemicals. It also highlights the value of integrating toxicology, analytical chemistry, bioinformatics, and epidemiology to better understand and address the complexity of real-world environmental exposures and their impact on human health.

O-7a-3

Case studies on chemical mixture risk assessment with a focus on human breast milk and human cord blood serum

ABSTRACT #66

Arslan Hashmi[1], Matthias Herzler[1], Sebastian Schmeisser[1]
[1]*German Federal Institute for Risk Assessment (BfR), Germany*

Throughout their lives, humans are simultaneously exposed to multiple chemicals, which requires individual chemical level data for mixture risk assessment (MRA) which is challenging. Within the EU project PANORAMIX, we evaluated existing MRA approach and conducted case studies. This presentation introduces different approaches to assess the risks of real-life coincidental co-exposure for human health and to evaluate their potential for regulatory applications. For this purpose, we performed two case studies, i.e., CS-1 & CS-2, focusing on potential health risks to infants and offspring due to postnatal and prenatal exposure to real-life coincidental chemical co-exposure respectively. For this work, we specifically focused on toxic effects on the developing reproductive and nervous systems (DRT & DNT) by employing a component-based approach (CBA) with the default assumption of additivity. The exposure information in both case studies was the starting point for an exposure driven MRA, based on HBM data, which was refined into hazard-based grouping at later stages. CS-1 followed a CBA by using human breast milk quantitative exposure data along with in vivo toxicological data from suitable animal and/or epidemiological studies. Contrarily, CS-2 used Chemical Mixture Calculator 2.0 (CMC 2.0), an online tool that provides data obtained with the help of emerging technologies such as suspect screening, exposure predictions, high-throughput toxicokinetic simulations, and in vitro bioactivity data pertinent to AOPs of DRT & DNT. In CS-2, suspect screening identified chemicals from cord blood serum , several CAGs were formed based on AOP (common

MIEs/KEs) information to identify similarly acting chemicals. The CMC 2.0 was used to provide the following single-substance information (1) maternal external exposure, (2) foetal steady-state plasma concentration, (3) in vitro points of departure, (4) HQs for each mixture component, (5) sum of the HQs of all chemicals to obtain an HI as a risk metric.

O-7a-4

Complex chemical mixtures across the environment-food-human continuum ABSTRACT #82

Anne Marie Vinggaard[1], Maria João Valente[1], Soléne Motteau[2], Maria Margalef[3], Maria König[4], Jean-Philippe Antignac[2], Marja Lamoree[3], Timo Hamers[3], Martin Scholze[5], Tina Kold Jensen[6], Beate Escher[4]
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[6]*SDU, DK*

Humans are continuously exposed to a wide range of chemicals from food, consumer products, and the environment—often as complex mixtures. Some of these mixtures may produce combined (mixture) effects that pose risks to human health. However, current chemical legislations do not sufficiently account for these mixture-related risks. The EU Green Deal project PANORAMIX aims to address these shortcomings, and several key outcomes from the project are presented here. We demonstrate how New Approach Methodologies (NAMs)— particularly in vitro bioassays—can provide added value in testing whole mixtures from real-life samples, including those derived from environment, food, and human sources. These NAMs have been applied to human cord blood samples from the Odense Child Cohort, enabling the linkage of in vitro assay outcomes to potential adverse health outcomes, with a focus on reproductive and neurodevelopmental endpoints. In addition, NAMs have supported component-based mixture studies which, when coupled with statistical modeling, confirm that large numbers of chemicals — even at low, real-life concentrations—can produce additive effects. Of particular concern is the observation that significant mixture effects may arise when chemicals at their individual POD levels (Point of Departure) are combined. To support practical application of this research, we developed a user-friendly, web-based platform: the Chemical Mixture Calculator 2.0. Designed for use by risk assessors, this tool enables preliminary mixture risk estimation. It integrates in vitro hazard data related to reproductive and neurotoxic effects with predicted human exposure levels for thousands of chemicals, allowing users to calculate hazard quotients and a cumulative Hazard Index for specific chemical mixtures. By integrating NAMs aligned with Adverse Outcome Pathways (AOPs), together with suspect screening approaches allowing extended exposure assessment and effect-directed analysis, PANORAMIX offers an innovative framework for the assessment of complex chemical mixtures and their potential impacts on human health.

Session 7b: Beyond Positive Results: Interpreting NAMs for Endocrine Disruptors

Chairs: *Elise Grignard (OECD) & Bambou Tan (L'Oréal)*

O-7b-1

OECD work on endocrine disruptors ABSTRACT #45

Elise Grignard[1]
[1]*OECD*

In the last years, the regulatory landscape has been changing with regard to requirements for data for evaluating potential endocrine disrupting properties of chemicals. Recent activity related to the European extension of Classification and Labeling regulation to include endocrine disruptors (ED) has extended to a proposal for consideration of adding an endocrine disruptors hazard class into the Globally Harmonized System of Classification and Labelling of Chemicals. To identify chemicals that may perturb 'traditional' estrogen, androgen, thyroid, and steroidogenesis (EATS) pathways, as well as other 'non-EATS' pathways, the OECD was given a mandate to evaluate and review the state of the science on the possibility to identify EDs, as such, under the GHS. In response, the

OECD convened an expert group to provide review and input on consultant-led reviews of the available methods to identify EATS and non-EATS EDs relevant to humans and the environment. While the conclusion of the group indicated there are methods available for many ED pathways, additional work is needed. In order to meet these new regulatory requirements, the development and validation of tools to test and assess the endocrine activity of chemicals are needed. The Integrated Approaches to Testing and Assessment (IATA) combine multiple sources of information to conclude on the toxicity of chemicals. Recently, the OECD has revised the IATA framework to increase its' usability and favor the use of New Approach Methods (NAMs) for regulatory decisions. The use of NAMs in IATAs is particularly relevant to the identification of EDs which is based on the WHO/IPCS definition, and thus requires mechanistic assays identifying chemicals that act through an endocrine mode of action, as well as, assays measuring apical, adverse effects in humans or wildlife populations.

O-7b-2

Hormone-active substance testing strategy based on NAMs: from standardized to more predictive in vitro methods

ABSTRACT #101

Nathalie Alépée[1]
[1]*L'Oréal Research and Innovation, Aulnay-sous-Bois, France*

The regulatory landscape for endocrine disruptor (ED) assessment is undergoing a crucial transformation. New hazard classes have been introduced by the Classification, Labelling and Packaging (CLP) regulation to identify chemicals with endocrine disrupting properties. These classes are based on the identification of an endocrine mode of action and the potential resulting adverse effects. Evolution unequivocally highlights the imperative to accelerate the development and validation of robust and predictive New Approach Methodologies (NAMs) linked to in vivo adverse outcome. We will detail here the ongoing development of innovative evaluation tools specifically designed to screen for endocrine disruption potential. Our approach places a particular emphasis on the synergistic construction and association of methods recognized by the OECD (Organization for Economic Co-operation and Development) and our proprietary internal tools. The development and application of in-house assays meticulously targeting key endocrine receptors (e.g., Estrogen Receptor (ER), Androgen Receptor (AR)) and other relevant molecular pathways are fundamental. While the application of validated NAMs, based on mechanisms of action, including Quantitative Structure-Activity Relationship (QSAR) models, in tubo binding assays and in vitro transactivation assays, offers immense promise, a significant challenge persists: accurately interpreting positive results. Moving beyond merely showcasing the existence of these methods, we will delve into the practical application of existing building blocks from the cosmetic industry. This involves leveraging validated NAMs, QSAR models, and both binding and transactivation assays to construct effective tiered assessment strategies. A particular emphasis will be placed on two proprietary methods crucial for the screening process. With this first example, we would like to empower collaboratively built strategies solutions to tackle this scientific challenge.

O-7b-3

Hazard assessment of substances on the endocrine (EATS) axes using human cell-based CALUX® reporter gene assays coupled to a metabolic module

ABSTRACT #97

Bram Brouwer[12], Clemence Budin[1], Tjalf de Boer[1]
[1]*BioDetection Systems BV*
[2]*VU University Amsterdam*

There is an increasing need/demand for risk assessment of chemicals using non-animal, new approach methods (NAMs). These NAMs need to be validated, are fit for purpose, are sensitive and reliable and have a high predictive value for toxic endpoints of interest, such as endocrine disruption. In the endocrine disruption field most interest is focused on the Estrogenic, Androgenic, Thyroid and Steroidogenesis (EATS) pathways. Several high sensitive, human based reporter gene

assays have been developed, such as CALUX reporter assays for nuclear receptors ERα (OECD-TG-455), AR (OECD TG-458) and TR (currently under OECD evaluation) as well as protein binding competition (TTR), enzyme inhibition (e.g., TPO, DIO) and cellular iodine transport (NIS) processes. In order to improve predictability even further we also include a human or rat liver S9 metabolic unit, with addition of cofactors for phase I and for phase I&II metabolism, to predict the metabolic fate and bioactivation/degradation of substances of interest. Results will be presented on a wide applicability of NAMs for EATS in the field of (agri) chemicals and cosmetics and environmental contaminants including their advantages, challenges and limitations, and a comparison is made between measured and predicted/expected results. Based on the results of these studies we developed and propose a standardized operation procedure for in vitro human-cell and -S9 based testing of substances for EDC activity with high predictive value.

O-7b-4

NAMs for thyroid hormone system disruption and their role in testing strategies
ABSTRACT #95

Timo Hamers[1], Kostja Renko[2], Eva Bay Wedebye[3], Marta Axelstad[3], Martin Scholze[4]
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[3]*National Food Institute, Technical University of Denmark, Kgs.Lyngby, Denmark*
[4]*Centre of Pollution Research and Policy, Brunel University, London, UK*

At present, no validated in vitro bioassays are available for the screening of chemicals for thyroid hormone system disruption (THSD) properties. The OECD Expert Group on Thyroid Disruption Methods (TDM EG) has evaluated the assessment reports for 18 mechanistic THSD in vitro bioassays that were standardized/characterized, optimized and tested for transferability from the original developers by EURL-ECVAM. Of these, five were identified as promising candidates to continue and complete the validation process. These include the DIO-1 assay, which measures inhibition of type 1 deiodinase activity in human liver microsomes based on the Sandell-Kolthoff reaction, and the FITC-T4 TTR-binding assay, which measures displacement of a fluorescently labelled FITC-T4 probe from its serum distributor protein transthyretin (TTR). Both assays have entered the OECD working program and are undergoing further transferability study within the PEPPER initiative. Meanwhile, additional NAMs for THSD-related molecular initiating events (MIEs) have been developed and transferred between laboratories within the EU-funded ATHENA and PARC projects. These include QSARs for MIEs involved in TH regulation, synthesis, distribution, and metabolism, as well as in vitro methods for the inhibition of thyroid hormone transmembrane transporter (THTMT) proteins in MCT8-, OATP1C1- or OAT4-overexpressing cell lines and choroid plexus organoids representing the blood-cerebrospinal fluid barrier. This presentation will provide an overview of the current validation status of these NAMs and highlight existing gaps in validated NAMs for other THSD-related MIEs. In addition, it will discuss the application of validated NAMs for mechanistic data gap-filling as part of a broader testing strategy for testing and assessment of chemicals for thyroid hormone system disruption, as proposed by the ATHENA project.

Wednesday, 1 July 2026
14:00 - 15:30

Session 8a: Systemic Effects Evaluation Using NAM Toolboxes – Real-World Cases

Chairs: *Helena Kandárová (CEM SAS) & Predrag Kukic (Unilever R&D)*

O-8a-1

Prioritization of ECHA chemicals using high-throughput in vitro screening methods
ABSTRACT #32

Matthias Wehr[1], Lukas Wijaja[2], Marlene Wedler[34], Christopher Wolff[5], Barira Islam[6], Tjalf de Boer[7], Bas Ter Braak[8], Shu Liu[3], Bob van de Water[2], Sylvia Escher[9]
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[5]*Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Germany*
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[8]*Toxys B.V., Oegstgeest, Netherlands*
[9]*Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM), Germany*

This case study aims to develop a prioritization and screening approach using New Approach Methodologies (NAM) to identify compounds, potentially harmful to human health. Model compounds were selected to investigate the effectiveness of high-throughput methods and their combinations for the prioritization of high toxicity compounds and deprioritizing of low toxicity. The selected compounds are labeled considering criteria for STOT-RE classification, which assesses systemic toxicity after repeated exposure. High-toxicity compounds are identified by adverse effects at concentrations below 10 mg/kg bw/d, while low-toxicity compounds show no observed systemic toxicity. The study incorporates four high-throughput in vitro assays, combined with kinetic models and machine learning techniques, to train a predictive model and evaluate its performance under varying conditions. Samples from in vitro primary human hepatocytes are sequenced using the TempO-Seq whole-transcriptome panel and phenomics data are measured in HepG2 using the CellPaintingPlus approach. Additionally, CALUX reporter gene assays and ToxProfiler fluorescent reporter assays are performed to further refine the methods with more targeted read-outs. Benchmark concentration analysis is used to quantify the response and interpret the results. The dataset comprises 61 compounds, balancing two classes of 32 high-toxicity and 29 low-toxicity compounds. The random forest model trained on all four assays achieved a sensitivity of 81.2% and a specificity of 89.7%. Notably, the CellPaintingPlus and TXG-MapR models contributed significantly to the predictive accuracy. However, some compounds were misclassified, highlighting the need to i) the integrate specific biological processes and ii) address technical aspects, such as clear definitions of relevant in vitro testing concentrations. The findings shows that biological activity profiles from a high-throughput in vitro screening battery can effectively prioritize compounds as systemically toxic. Future work should focus on enhancing the in vitro concentration range and validating the model with external datasets to improve its applicability in regulatory contexts.

O-8a-2

Building confidence in non-animal safety frameworks for systemic toxicity ABSTRACT #12

Predrag Kukic[1]
[1]*Unilever*

Performing risk assessments for repeated-exposure systemic toxicity without animal data is a significant challenge. Yet, using exposure-led tiered frameworks enables us to meet this challenge today, especially in the area of consumer products. These frameworks include established approaches such as exposure-based waiving and read-across. Where these are either not applicable or insufficient to assure safety, a variety of in silico, in chemico, and in vitro techniques can be used to inform safety decisions. We have used such techniques to develop a combined non-animal toolbox which is designed to be protective for systemic toxicity. This toolbox includes physiologically-based kinetic modelling to predict internal exposures, and 3 bioactivity platforms: high-throughput transcriptomics in 3 cancer cell lines, a cell stress panel, and in vitro pharmacological profiling to give broad coverage across many different toxicity modes of action. We evaluated the level of protection provided by this toolbox using 40 case study chemicals and associated exposure scenarios. In most cases (>90%), the toolbox was protective of human health, similar to the level of protection offered by traditional animal-based assessment approaches. Furthermore, to understand to which degree this protection level is sensitive to changes in the choice of bioactivity platforms, the same chemicals were tested in a separate toolbox comprising of 2

bioactivity platforms: high-throughput phenotypic profiling and high-throughput transcriptomics across 10 human-derived cell lines from different organs, primarily hTERT cell lines. By comparing these 2 distinct toolboxes, we expect to establish the degree of replication between platforms in order to qualify/validate the approach for regulatory acceptance. This approach increases our confidence in the robustness of non-animal toolboxes enabling their application to safety decision making for systemic toxicity and prevents unnecessary animal use.

O-8a-3

Next generation risk assessment (NGRA): overcoming barriers in the food sector

ABSTRACT #15

Danilo Basili[1], Elena Reale[1], Myriam Coulet[1], Thomas Stroheker[1], Benjamin Brinon[1], Yann Ratinaud[1], James Holzwarth[1], Patrick Descombes[1], Marjorie Guitard[1], Alexandre Panchaud[1], Gina Montoya[1]
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Next Generation Risk Assessment (NGRA) provides a transformative approach to chemical safety evaluation. The cosmetic industry has widely adopted NGRA methodologies following the 2013 ban on animal testing, which accelerated the use of alternative assessment strategies [1]. In contrast, the food sector has been slower to adopt these advancements, often relying on traditional methods that may not be human relevant. Recent EU initiatives to phase out animal testing signal a critical shift, highlighting the need for the food industry to embrace modern risk assessment practices. Despite the rapid development of New Approach Methodologies (NAMs), a significant challenge remains in integrating them into toolboxes to enhance decision-making confidence, especially for assessing systemic effects that require comprehensive biological coverage [2,3]. Building on existing public knowledge, we assembled a NAM toolbox for hazard characterization of systemic effects to explore its efficacy in driving safety decisions for food-relevant substances spanning chemical contaminants and chemicals used in food. The toolbox included NAMs for cell stress profiling, in vitro pharmacological profiling and high-throughput transcriptomics in three human cell lines, which were used to derive points of departure (PoDs). General Physiologically-Based Kinetic (PBK) models estimated internal exposure (Cmax) based on relevant exposure scenarios, categorized as high or low concern. By calculating chemical PoD-Cmax Ratios and comparing them to historical safety levels, we successfully classified high-risk chemicals, demonstrating the NGRA framework's ability to inform safety decisions without animal testing. In addition, this work also identifies challenges that need to be addressed, such as distinguishing between adaptive and adverse effects, and evaluating whether the NAM toolbox offers comprehensive toxicological coverage relevant to the food domain.

O-8a-4

ID2STOT – A workflow for the classification of systemic toxicities based on concentrations of bioactivity referenced by internal doses

ABSTRACT #72

Philipp Demuth[1], Eric Fabian[1], Anastasia Weyrich[1], Susanne Kolle[1], Lihua Deng[1], Karin Wiench[1], Robert Landsiedel[1]
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The ID2STOT workflow, which was developed by BASF in the context of the EPAA-Designathon for Human Systemic Toxicity, aims to classify chemical compounds regarding specific target organ toxicity (STOT) based on bioactivity concentrations referenced by internal doses. The workflow, that addresses repeated exposure (RE) leading to STOT, is applied to a set of compounds based on the predefined list of EPAA-Designathon substances with low, medium and high hazard as well as diverse modes of action. The goal is a regulatory-applicable NAM-based approach that utilizes toxicokinetic data to incorporate the internal availability of a compound and thereby quantifies systemic toxicity to classify the hazard for STOT RE. The workflow follows a defined structure: First, a read across is applied to identify the nearest neighbors and collate their existing experimental in vitro data which is used to guide the following bioactivity assessment of the test substances. Second, RNA-sequencing is conducted to quantify dose-dependent transcriptomic changes,

based on which a concentration of bioactivity (CoB) is derived. Furthermore, the transcriptomic data is applied to substantiate or improve the read across and identify potential higher tier in vitro assays. In parallel, the GHS STOT RE class limits, which represent external doses, are translated to compound specific internal dose limits (IDlimits) by applying physiology-based toxicokinetic (PBTK) modelling with experimentally and in silico derived input parameters. Finally, the CoB is compared with IDlimits to classify the substances for systemic toxicity. Uncertainty and variability within the toxicokinetic and toxicodynamic assessments are addressed by applying Monte-Carlo and Benchmark-Dose modelling. This NAM-toolbox approach is applied to 15 substances, allowing for a categorization into three hazard classes (low, medium, and high) and a subsequent evaluation of the workflow. The project demonstrates the feasibility of using the in silico and in vitro based ID2STOT approach for STOT RE classification.

Session 8b: Towards robust and reliable in vitro models for Developmental and Reproductive toxicology

Chairs: *José M. Horcas Nieto (Toxys B.V., The Netherlands) & Amer Jamalpoor (Toxys B.V., The Netherlands)*

O-8b-1

Validation of the ReproTracker assay for regulatory use following ICH SH5 (R3) guideline
ABSTRACT #4

José M Horcas-Nieto[1], Jing Fang[1], Sabine Hartvelt[1], Connor Philippo[1], Gaonan Zhang[1], Tom Zwetsloot[1], Giel Hendriks[1], Amer Jamalpoor[1]
[1]*Toxys B.V., Leiden BioScience Park, The Netherlands*

By optimizing validation requirements and promoting regulatory confidence in new approach methodologies, ICH S5 (3R) is paving the way for the acceptance of alternative models for developmental and reproductive toxicity (DART) testing. The ReproTracker assay is a human induced pluripotent stem cell (hiPSCs)-based system in which hiPSCs are differentiated towards three germ layer-specific cell types: cardiomyocytes (mesoderm), hepatocytes (endoderm), and neural rosettes (ectoderm). This system assesses teratogenic potential by tracking alterations in biomarker expression and morphological disruptions in the differentiated cells upon compound exposure. Here the ReproTracker was validated using the compounds listed in the ICH S5 (3R) guidelines. Following identification of human developmental toxicants, we also assessed the clinical relevance of concentrations detected as potentially teratogenic by investigating their correlation with their corresponding human therapeutic Cmax. In this study, ReproTracker correctly classified 28 out of the 32 reference compounds, highlighting the assay's potential for DART testing. Two of the teratogenic compounds (DNA modifying agents: 5-Fluorouracil and methotrexate) exhibited a highly cytotoxic profile leading to very low concentrations (less than 1 and 0.01 µM, respectively) being tested as top doses, leading to false negative results. 17 out of 24 known in vivo teratogens were identified at concentrations equal to or below their respective human therapeutic Cmax. 7 compounds were detected within a 10- to 40-fold range above it, while two of these were limited by the lowest tested concentration in the assay. Overall, ReproTracker demonstrated a high predictability (96%) and sensitivity (90%), highlighting the potential of the assay in teratogenicity assessment. These results showcase the potential of ReproTracker to predict teratogenicity of pharmaceuticals and position the assay at the forefront of in vitro DART testing. This validation following ICH S5 guidelines provides a solid foundation for further validation and integration of ReproTracker into safety assessment frameworks.

O-8b-2

Dose response modelling and transferability of ReproTracker- an industry perspective
ABSTRACT #27

Kathryn Wolton[1], Jade Houghton[1], Magdalena

Sawicka[1], Iris Muller[1], Theo Timmerbacka[1], Alistair Middleton[1], Beate Nikol[1], Gopal Pawar[1], José M. Horcas-Nieto[2], Connor Philippo[2], Amer Jamalpoor[2], Sabine Hartvelt[2], Remco Derr[2], Giel Hendriks[2], Annabel Rigarlsford[1], Lucy Bull[1], Mathew Van de Pette[3], Luke Flatt[2]
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[2]*Toxys B.V., De Limes 7, 2342 DH Oegstgeest, Netherlands*
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A framework for making non-animal safety decisions for systemic toxicity, (including developmental and reproductive effects) has been developed which uses physiologically-based kinetic modelling and in vitro bioactivity assays to provide bioactivity:exposure ratios (BERs). The framework has been evaluated using 37 benchmark compounds across 49 exposure scenarios and has shown promise in providing protective safety decisions for the majority (17/18) of high DART-risk scenarios. One of the DART-specific bioactivity assays included in the framework is ReproTracker® (Toxys). ReproTracker® is a human stem cell-based assay that assesses perturbations in stem cell differentiation into cardiomyocytes, hepatocytes and neural rosettes. In addition to assessing the performance of the framework as a whole, we have assessed the reproducibility of ReproTracker® high throughput transcriptomics dose response data and the inter-lab transferability of this assay, to increase confidence in its use for safety decision making. To this end, we have tested an additional 40 chemicals in 3 independent ReproTracker® experiments and assessed the impact of different dose-response modelling methods on the points of departure obtained. We have also performed a transferability study of ReproTracker® across two independent laboratories (Toxys, NL and Unilever, UK). This involved testing 10 blinded chemicals in both laboratories. Identical data analysis pipelines were applied to both result sets and compared for evaluations of inter-lab reproducibility. The results from these analyses will be shared, and the strengths and limitations different approaches to calculating points of departure will be discussed. These types of analysis are crucial in understanding the overall robustness of the ReproTracker® assay and can be applied to other components of our NAM toolbox to ensure confident safety decision making.

O-8b-3

Supporting NAM-based hazard characterization through computational biokinetic modeling
ABSTRACT #76

Styliani Fragki[1], Susana Proenca[1], Lara Lamon[1], Stephan Schaller[1], Marco Siccardi[1]
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Physiologically based kinetic (PBK) models are critical for simulating the in vivo behavior of chemicals using physiological and compound-specific parameters. In parallel, in silico mass balance models predict compound distribution in vitro by incorporating system-specific and physicochemical properties. Together, these biokinetic approaches link in vitro unbound concentrations to in vivo plasma or tissue levels, supporting external dose prediction in Quantitative In Vitro to In Vivo Extrapolation (QIVIVE). Biokinetic modeling plays a central role in Next Generation Risk Assessment (NGRA), translating in vitro effect data into in vivo dose estimates. A tiered assessment strategy is essential to make NGRA more efficient, flexible, and transparent—reserving the use of more complex models for situations with high uncertainty and narrow safety margins. Therefore, it is important to understand how QIVIVE predictions evolve across assessment tiers—from streamlined, high-throughput PBK models requiring minimal physicochemical and ADME data, to more mechanistically detailed and data-informed models. Developmental and reproductive toxicity (DART) testing, traditionally reliant on animal studies, is a key area for advancing new approach methodologies (NAMs). This presentation highlights the application and integration of both in vitro and in vivo biokinetic models for translating DART-NAMs, with a particular focus on pregnancy. It will also showcase the application of the free and open-source Open Systems Pharmacology (OSP) suite for maternal-fetal QIVIVE modeling, discuss approaches for parameterizing chemical placental transfer, and identify key

data gaps that remain to be addressed. Case studies, including developmental neurotoxicity (DNT), will be used to demonstrate QIVIVE application in a tiered risk assessment. Resulting Human Equivalent Doses (HEDs) are compared to traditional animal safety thresholds, supporting evaluation of relative protection and informing the application of uncertainty factors in NGRA.

O-8b-4

From synapse to signal: human brain MPS as next-generation tool for DNT testing

ABSTRACT #89

Lena Smirnova[1]
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Background The assessment of developmental neurotoxicity (DNT) remains one of the most pressing yet under-addressed needs in regulatory toxicology. Traditional animal models often fail to recapitulate human-specific features of brain development, limiting their predictive value. Recent advances in human induced pluripotent stem cell (iPSC)-derived brain microphysiological systems (bMPS)—including organoids, assembloids, and organ-on-chip platforms—offer a transformative alternative by integrating multiple neural cell types, including microglia and oligodendrocytes, and capturing critical neurodevelopmental events such as synaptogenesis, myelination, and neuroinflammation. Methods and Results Our recent work exemplifies how these complex models can be leveraged to assess chemical-induced perturbations of neurodevelopment. We demonstrated the utility of bMPS in detecting subtle functional disruptions caused by chronic low-dose exposures to environmental toxicants such as domoic acid and metal mixtures. Functional readouts—including calcium imaging, high-density microelectrode array (HD-MEA) recordings, and immediate early gene expression—revealed dose- and time-dependent impacts on synaptic plasticity and network dynamics, correlating with cognitive endpoints such as learning and memory. Further, by the integration of genetic risk factors (e.g., 16p11.2 deletion) and immune challenges in bMPS, we elucidated gene–environment interactions relevant to autism spectrum disorder, highlighting the potential of these systems to model interindividual variability. Discussion and Conclusions This presentation will discuss key challenges and solutions in the validation of bMPS for DNT testing, including the definition of applicability domains, standardization through Good Cell Culture Practice (GCCP), and the use of reporter lines for real-time monitoring of developmental processes. By aligning with international efforts toward structured validation of New Approach Methodologies (NAMs), our work illustrates how bMPS can serve as advanced, human-relevant tools in tiered DART testing strategies, ultimately improving public health protection while reducing reliance on animal testing.

O-8b-5

Advancing regulatory acceptance of NAMs for developmental toxicity: insights from ICH S5(R3)

ABSTRACT #10

Peter Theunissen[1], Puck Roos[1]
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The revision of ICH S5 to S5(R3) marked a pivotal step in the regulatory recognition of new approach methodologies (NAMs) for developmental and reproductive toxicity (DART) testing. For the first time, an international guideline formally included provisions for the qualification and regulatory use of NAMs to assess embryo-fetal developmental (EFD) toxicity, with the potential to reduce animal use and increase human relevance. Under ICH S5(R3), to replace pivotal DART studies, NAMs must be qualified within a well-defined context of use (CoU), which includes the chemical applicability domain, biological mechanisms covered by the assay, and the specific nonclinical endpoints the assay is intended to predict—particularly malformations and embryo-fetal lethality (MEFL). Despite this framework, the uptake of NAMs in regulatory submissions remains limited. Developers and industry stakeholders often perceive the qualification process as insufficiently transparent and administratively demanding, while regulators observe a disconnect between the intended role of NAMs—as components in a weight-of-evidence (WoE) approach—and expectations

for one-to-one replacement of in vivo studies. Additional barriers include limited regulatory experience, lack of clarity on procedural pathways, and reluctance to share proprietary data. To address this impasse, efforts are underway within European regulatory bodies to support early dialogue with NAM developers (e.g., via the EMA's Innovative Task Force), promote voluntary data sharing (VDS), and develop specific guidance for organ-on-chip (OoC) and other NAM platforms. Lessons learned from the S5(R3) revision highlight the critical need for clear communication, stakeholder alignment, and structured regulatory support to unlock the full potential of NAMs in DART testing.

Wednesday, 1 July 2026
17:00 - 18:30
Session 9a: Multimodal Modelling of the Gut Compartment - NAMs for Food Safety

Chairs: *Giorgia Del Favero (Department of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Vienna, Austria) & Thomas Hartung (Center for Alternatives to Animal Testing (CAAT), Johns Hopkins University, USA)*

O-9a-1

Developing tools to assess the intestinal barrier integrity at the nanoscale
ABSTRACT #57

Janice Bergen[12], Claudia Iriarte-Mesa[3], Francesco Crudo[1], Doris Marko[1], Freddy Kleitz[3], Giorgia Del Favero[1]
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Background and Objectives: Maintenance of barrier function is one of the fundamental features of the intestinal compartment. A highly specialized epithelium covered with mucus ensures selective permeability and mechanical stability, supporting nutrient absorption, peristaltic transit, and protection toward potentially noxious xenobiotics. Yet, exposure to chemical mixtures through lifestyle and diet can challenge this system, and cell culture models capable of describing these delicate interactions are essential for hazard characterization. Materials and Methods: To grasp toxicity mechanisms targeting the intestinal barrier, we built on the knowledge developed for the bioactivity profiling of nanomaterials. Adapting shape and surface functionalization for drug delivery purposes, we gained precious insights into physical-chemical parameters enabling penetration through the mucus and modulation of paracellular permeability. Tuning size, aspect ratio, hydrophobicity/hydrophilicity, and/or surface charge, a library of silica-based materials was synthesized [1;2] and used according to the capacity to cross the mucosal barrier and interact with tight junction proteins like Claudin-4 and Zonula occludens-1. Results and Discussion: To probe the functionality of the cell monolayer at the nanoscale, we recently proposed an implementation of the human intestinal co-culture model Caco-2/HT29-MTX-E12 integrating SiO2-based mesoporous nanorods (bacteria-like 200x450 nm; small rods 35x160 nm) [3]. Here, we extended the use of nanomaterials as "molecular scissors" to selectively evaluate barrier function. With this approach, we aim at reproducing aspects of the mechanical load exerted within the intestinal lumen by the microbiome and the mass of the food chyme. Comparing cell-cell adhesion and cells-nanomaterials interactions with or without prior exposure to foodborne contaminants, we interpreted variations as early signs of impairment. This workflow enables the description of detrimental effects at the cell or mucus level that might not be evident using exclusively the cell co-culture model and contributes to the development of systems that efficiently support the screening of food contaminants in vitro.

O-9a-2

Computational toxicology unveils receptor-mediated effects of food toxins at the gut level: A structural perspective for food safety assessment ABSTRACT #22

Lorenzo Pedroni[1], Florinda Perugino[1], Chiara Dall'Asta[1], Luca Dellafiora[1]
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The presence of toxins in food may severely affect the health and well-being of humans worldwide, and the gut represents either the first site of offence or the upstream barrier of defense. A body of evidence has associated the dietary exposure of certain toxins with food-borne diseases. Also, a growing amount of evidence has begun strengthening the linkage between the exposure of certain food toxins to adverse effects not strictly considered of food origin before (like some neurotoxic insults, prodromic of neurodegeneration; e.g. ref. [1]). Regardless of the adverse effects and the underlying mechanisms, many of them may originate from the interaction between food toxins and receptors expressed in the gut epithelium, including but not limited to those typically associated with food flavors perception in the upper part of gastrointestinal tract (e.g. bitter and odor receptors). Taking advantage of recent breakthroughs in 3D protein modelling brought by AlphaFold and Boltz [2], and based on previous work showing the applicability of 3D molecular modelling to investigate the mechanistic underpinnings of gut receptors [3], the present paper computationally delves into the "food toxicome" available so far. Database for food toxins like EFSA's OpenFoodTox and T3DB are browsed and screened against the gut "receptome", mapped integrating expression profiles as per Protein Atlas (https://www.proteinatlas.org) and 3D protein structure modelling based on docking, molecular dynamics simulations and machine learning. By modelling gut receptor-toxin interaction at an atomistic resolution, the study provides either a knowledge-based prioritization of systems and mechanisms of action for further analysis or extends the mechanistic understanding of already known modes of action. The integration of 3D modelling with big data availability is outlined as an effective framework of analysis in the context of NAMs for food safety, raising understanding at a precise structural and mechanistical level.

O-9a-3

Advances in microbiome-competent PBK modeling to quantitatively predict the toxicity of gut microbial biotransformation products. ABSTRACT #71

Georg Aichinger[1]
[1]*ETH Zurich*

Xenobiotic metabolism in the liver is classically considered the defining process of toxicokinetics, but gut microbiota recently emerged as significant contributors to chemical biotransformation. However, the physiological relevance of such reactions often remains unclear. Microbiome-competent PBK models have emerged as tools to quantify the toxicokinetics of gut microbial metabolites and thus facilitate their toxicological evaluation, including considerations of inter-individual variability. We thus established such models for different chemicals of interest, accounting for metabolite formation in the gut, gastrointestinal absorption, partitioning to organs of interest, glucuronidation in the liver and small intestine, urinary excretion, as well as QSAR-predicted enterohepatic circulation. For parameterizing the gastrointestinal compartment, we optimized a fecal fermentation assay, using stool samples from healthy human donors to measure microbial biotransformation kinetics by LC-MS/MS. The models were evaluated against human pharmacokinetic data and then used for quantitative in vitro to in vivo extrapolation of toxicity in mammalian cell cultures. Besides the direct read-out for microbiome-related hazard characterization, the developed methodology can now be used as blueprint strategy for microbiome-related chemical risk assessment.

O-9a-4

AI-driven advances in gut-competent NAMs for food safety assessment ABSTRACT #35

Thomas Hartung[12]
[1]*Johns Hopkins University*
[2]*University of Konstanz*

Background and Objectives The increasing global demand for safe food, combined with pressures from climate change and chemical complexity, highlights the limitations of animal-centric food safety testing. Naturally occurring contaminants, human-made chemicals, and microbiome-mediated transformations present dynamic challenges for risk assessment. New Approach Methodologies (NAMs) for gut toxicity are underdeveloped due to the digestive tract's structural complexity. This contribution aims to demonstrate how artificial intelligence (AI) enables the development, integration, and interpretation of gut-relevant NAMs in food toxicology. Materials and Methods We present recent advances in high-throughput in vitro systems and in silico models simulating gastrointestinal (GI) environments. These include co-cultures of intestinal epithelial cells with immune and mucus-producing cells, exposure platforms incorporating microbiota, and gut-on-chip technologies. On the computational side, we highlight AI-enhanced quantitative structure-activity relationship models (e.g., RASAR), probabilistic risk modeling, and deep learning for multi-modal data integration, including omics and imaging. Results AI-driven models demonstrated strong predictive performance for various food-relevant endpoints, outperforming traditional approaches in identifying hazards from structurally diverse xenobiotics [OBJ]. Case studies show that incorporating transcriptomics and microbial biotransformation data into mechanistic models improves interpretation of mixture toxicity and inter-individual variability. Nanoparticle-laden intestinal co-cultures combined with AI-based image and barrier function analysis enabled sensitive detection of epithelial disruption. Discussion and Conclusion AI enhances both the scalability and mechanistic insight of gut-relevant NAMs, addressing key data gaps in food safety. By incorporating explainable AI frameworks and aligning with regulatory principles such as TREAT (Trustworthiness, Reproducibility, Explainability, Applicability, Transparency), these tools are moving toward practical application in regulatory toxicology. Continued progress will require interdisciplinary collaboration, high-quality data sharing, and flexible validation strategies to modernize food safety assessment while minimizing animal use.

O-9a-5

AOP-aligned NAMs to link gut microbial events to liver outcome: case study on food nanomaterials.

ABSTRACT #8

Laure-Alix Clerbaux[1]
[1]*UCLouvain, Brussels*

Background and Objectives The gut microbiota contributes to human health through the production of key metabolites such as short-chain fatty acids, secondary bile acids, vitamins, and neurotransmitters. These microbial metabolites influence the host health via notably the well described gut-liver axis. Despite this, the role of microbial metabolism is often overlooked in toxicological assessments. Food additives, particularly nanomaterials (NMs) like synthetic amorphous silica (SAS), may alter microbial activity, raising concerns about downstream health effects. Embedding human-based New Approach Methodologies (NAMs) within the Adverse Outcome Pathway (AOP) framework offers a promising route to mechanistically link microbiota alterations with liver outcomes to support next-generation risk assessment (Clerbaux, ALTEX, 2025). Materials and Methods: AOP was developed to depict underlying microbiota-host mechanisms linking gut microbial events to liver outcomes. NAMs were proposed for the different KEs to be measured, with a specific consideration for NAMs linked to physicochemical characterization of nanomaterials. Specific bacteria were exposed to in vitro well dispersed and digested (INFOGEST) SAS metabolite production were quantified with a stable isotope-labeled assay as well as degree of liver receptor activation and of inflammatory response. Results SAS digestion and dispersion significantly altered nanoparticle agglomeration states, which in turn affected bacterial metabolism. In vitro digestion led to disaggregation of SAS, emphasizing the need to assess NM toxicity under physiologically relevant conditions. The use of standardized protocols was critical for reproducibility. An in vitro assay allows to assess specific liver receptor activation by microbial metabolites and the release of pro-inflammatory cytokines. Discussion and Conclusion Integrating data produced by human-based NAM within an AOP represents a crucial step towards establishing causality in microbiota-driven liver toxicity of food nanoparticles. This study could pave the

way to position further gut microbial metabolism in nanotoxicology.

Session 9b: NAMs in Cardiac Safety

Chairs: *Erika Ferrari (BiomimX Srl) & Arno Gutleb (Invitrolize)*

O-9b-1

UHearts: mechanically stimulated beating heart-on-chip models to advance cardiac safety
ABSTRACT #52

Roberta Visone[1], Caterina Pernici[1], Alberto Mantegazza[2], Marco Mondini[1], Marco Rasponi[2], Erika Ferrari[1], Paola Occhetta[12]
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Cardiotoxicity remains a major concern in the preclinical phase of drug development. To address this, we developed uHeart(1), a functional 3D cardiac microtissue model within a mechanically actuated organ-on-chip (OoC) platform(2) and we validated it for cardiac safety by following ICH S7B guidelines. This model combines hiPSC-CMs, either atrial and/or ventricular, with human fibroblasts (75:25 ratio) embedded in fibrin and cultured in 3D under physiological uniaxial cyclic strain (10%,1Hz). uHeart was qualified for QT-prolongation and pro-arrhythmia detection using 11 drugs listed in the CiPA(3). The model was further exploited for evaluating the effect of 19 reference compounds that affect contractility by acting on calcium homeostasis, on sodium channel and on the sarcomere. Mechanical stimulation ensured a reproducible development of highly interconnected spontaneously beating and synchronized microtissues in 7 days (yield~90%; coefficient of variation in the beating period-BP<25%). Transcriptomic and immunofluorescence analyses confirmed chamber-specific phenotypes and enhanced maturation, with elevated expression of structural (e.g. MYL2,MYL7,MYH7,MYH6) and ion-channel related genes (e.g. SCN5A,KCNH2,CACNA1C). The analyses on field potential (FP) (i.e., BP, spike amplitude, FP-duration) revealed that uHeart responded to drugs acting on single and multiple ion channels, demonstrating 83.3% sensitivity and 100% specificity in predicting QT-prolongation. Moreover, uHeart showed a superior outcome respect to 2D models in detecting arrhythmic events. The analyses on contractile parameters (e.g., BP, contraction time-CT, relaxation time-RT, contraction amplitude-CA, contraction velocity-CV) highlighted that uHeart has 65% sensitivity and 100% specificity in predicting inotropic effects. Notably, uHeart outperformed traditional 2D models in detecting the effects of drugs targeting the sarcomere, highlighting its enhanced relevance for application in more narrowed context of uses (COUs). Overall, uHeart is a versatile and human-relevant model for cardiotoxicity screening. Future work aims to incorporate vascular and immune components and assess structural cardiotoxicity and novel therapeutic modalities, advancing towards animal-free safety testing aligned with regulatory expectations.

O-9b-2

Beyond CiPA: emerging electrophysiological platforms in cardiac safety testing
ABSTRACT #83

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Electrophysiological assays based on human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are increasingly established in cardiac safety pharmacology and preclinical arrhythmia risk assessment. Microelectrode array (MEA) technology has proven to be a robust and scalable platform for monitoring cardiac electrophysiological activity, and has been widely evaluated in multi-site validation studies (e.g., Blinova et al., 2018) as part of regulatory initiatives such as CiPA. Despite this progress, conventional 2D hiPSC-CM monolayer models exhibit known limitations, including limited sensitivity to subtle

drug-induced effects and reduced capacity to capture complex proarrhythmic mechanisms. To address these challenges, innovative assay technologies have been developed, such as the integration of the Foresee Biosystems' laserporation system (IntraCell). This approach allows high-precision intracellular recordings without classical invasive methods, providing detailed electrophysiological insights into drug-induced changes at a single-cell resolution (e.g. Schäfer et al.,2022). Concurrently, the recent application of meshMEA technology offers an unprecedented opportunity to characterize electrophysiological properties of 3D heart-forming organoids (HFO with distinct cellular layering (Drakhlis et al., 2021). We are currently exploring these layered organoids to assess electrical signal propagation across distinct cardiac tissue layers, potentially capturing clinically relevant transmural gradients. These methodological innovations support a more differentiated assessment of electrophysiological phenotypes and contribute to improved assay robustness and translational relevance. They also open opportunities to detect compound effects that may remain undetected in classical extracellular readouts, particularly in the context of borderline or mechanism-specific proarrhythmic risk. Here, we will provide an overview of current electrophysiological assay formats, discuss their respective strengths and limitations, and introduce selected recent developments in the field to improve predictive validity and mechanistic insight in preclinical cardiac safety testing.

O-9b-3

Cardiotoxicity adverse outcome pathway network: towards mechanistic and quantitative modelling
ABSTRACT #391

Luiz Ladeira[1], Devon A. Barnes[2], Rosalinde Masereeuw[2], Liesbet Geris[13], Bernard Staumont[1]
[1]*University of Liège*
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Chemical-induced cardiotoxicity arises from diverse molecular disturbances that propagate across biological scales to produce organ-level dysfunction, but current assessments still rely on narrow endpoints. The Adverse Outcome Pathway (AOP) framework offers a structured way to connect molecular initiating events, key events (KEs), and adverse outcomes in a high-level overview of mechanisms. Extending linear AOPs to an integrated AOP network improves coherence and biological relevance, highlighting cross-cutting KEs, exposing systemic contributors, and providing a mechanistic basis to design animal-free testing strategies. We aimed at exploring the OECD AOP-Wiki to develop an AOP network for cardiotoxicity, focusing on identifying KEs impacting the heart. Thirteen AOPs yielded a network of 64 nodes, being 11 molecular initiating events (MIE), 48 KE, 5 adverse outcomes (AO), and 96 documented key event relationships. Oxidative stress was the most connected, being the most common KE towards AOs. Mitochondrial dysfunction and decreased cardiac contractility were recurrent mediators bridging diverse initiators to cardiac AOs. Extra-cardiac integrators, such as KEs happening in the kidneys, highlight systemic mechanisms towards cardiotoxicity. Overall, 53.2% of KERs had high/strong evidence, while only 38.3% reported any quantitative understanding. In addition, we analyzed the methods reported in AOP-Wiki and built a methods catalogue that can be interactively explored in a FAIR-compliant version of the network. These insights and resources help prioritize endpoints for an animal-free, human-relevant test battery and pinpoint high-impact gaps for quantitative AOP development. The KE-assay catalogue and the interactive model translate the analysis into actionable resources for Integrated Approaches to Testing and Assessment and provide a scaffold for future quantitative integration and regulatory use.

O-9b-4

Could succination lead to cardiotoxcity
ABSTRACT #94

Afaf Fizazi[1]
[1]*Umons*

Abstract Cardiovascular diseases (CVDs) are a major global health challenge, leading to high rates of illness and death. Conditions like heart failure, atherosclerosis, and ischemic cardiomyopathy not only shorten lives but also seriously affect the well-being of those who live with them. To improve treatment options, we

need to better understand the molecular processes that go wrong with these diseases. SUMOylation is a type of post-translational modification where SUMO proteins attach to other proteins to regulate their activity. This mechanism is especially important in the heart. For example, SUMO1 a member of the SUMO protein family, helps regulate SERCA2a, a pump that controls calcium levels and ensures the heart muscle relaxes properly after each contraction. When SUMO1 function is disrupted, SERCA2a's function is also affected, which can contribute to cardiotoxicity. In our study, we looked at how another process called succination, which occurs when the Krebs cycle metabolite, fumarate, is highly concentrated in cells and reacts with proteins, which might interfere with SUMO1's ability to modify SERCA2a. By enriching AC16 cardiomyocytes with sodium fumarate, we observed changes in metabolism using 1H-NMR spectroscopy. Our findings through Western blotting suggest that succinated SUMO1 was less effective at modifying SERCA2a. This suggests that fumarate accumulation could impair calcium metabolism in cardiomyocytes, potentially leading to toxic effects. Our findings reveal a new link between two protein modifications, succination and SUMOylation, and how they may work together to affect heart health. This insight opens the door to new ways of thinking about and possibly treating cardiovascular diseases in the future.

O-9b-5

Mechanistic class-specific cardiac safety profiling using a microphysiological system mimicking native heart biomechanics
ABSTRACT #3

Bettina Lickiss[1]
[1]*innoVitro GmbH*

As the pharmaceutical industry shifts toward non-animal methods (NAMs) for preclinical safety assessment, there is a growing need for in vitro systems that are both physiologically relevant and predictive of human cardiac responses that should not come at the expense of throughput and scalability. In the light of this demand, we demonstrate the validation of a microphysiological 96-well platform designed to replicate key biomechanical features of the human myocardium through ultra-thin, flexible PDMS membranes. These membranes support the maturation and stable function of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), enabling the detection of pharmacologically relevant contractile responses. To qualify the utility of this platform in cardiac safety testing, we evaluated a wide range of compounds from distinct mechanistic classes, including ion channel modulators, tyrosine kinase inhibitors (TKIs), anthracyclines, molecularly targeted therapies, and conventional small molecules. Key cardiac functional parameters - including beat rate, contraction force, contraction duration, and arrhythmic events – were assessed. The platform demonstrated the detection of class-specific phenotypic signatures with high consistency and translatability, including both positive and negative inotropic effects, conduction disturbances, and arrhythmogenic properties. These findings highlight the system's ability to discriminate compound-specific cardiac liabilities, offering mechanistic insight and translational relevance. Overall, the data support the use of this biomimetic, human-cell-based platform as a versatile and sensitive NAM for early-phase cardiac safety assessment. While most of the data used in this study was collected in public-private-partnership ring trials, we advocate the establishment and consistent application of such efforts for thorough qualification of NAMs considering the significant evolution of regulatory frameworks.

Wednesday, 1 July 2026 18:30 - 19:30
Session 10b: State of the art of in vitro DART assays: The bigger picture

Chairs: *Darren Kidd (Labcorp, Harrogate, UK) & Amer Jamalpoor (Toxys B.V., The Netherlands)*

O-10b-1

A defined approach based on NAMs for assessing teratogenicity
ABSTRACT #33

Matthew Burbank[1], Florian Gautier[1], N. J. Hewitt[2], Audrey Noel-Voisin[1], Nazanin

Golbamaki[1], Romain Grall[1], Marc Léonard[1], Léopold Carron[1], Anne Riu[1], Sylvie Emery[1], Anne Detroyer[1], Typhaine Bringel[1], Noémie De Crozé[1], Laurent Guillet-Revol[1], Gladys Ouédraogo[1]
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Cosmetic relevant ingredients must be evaluated for their safety for their intended use. For more than two decades, the use of animal studies for such evaluations has been banned, and as a result the cosmetics industry has been a major contributor to the development of non-animal new approach methodologies (NAMs). The development of in vitro developmental and reproductive toxicity (DART) models is challenging. In this work, we have reviewed NAMs used to assess the safety of chemicals and investigated their coverage of DART using Adverse Outcome Pathways (AOPs) and other biomarkers. Screening tools as in silico QSAR model, transcriptomics, and pharmacology profiling can be used to both generate and test hypotheses about mode of action of new compounds. Moreover, the increasing availability of induced pluripotent stem cells, fish embryos provide greater range of biological models that closely mimic the human biology relevant for addressing a specific hypothesis. The complementarity of three NAMs, alone and in combination, for their ability to assess the teratogenic potential of a panel of chemicals. DART decision tree model, the devTox assay and the Zebrafish Embryotoxicity Test were used. The starting training set of 85 test chemicals comprised 46 classified as non-teratogenic and 39 classified as teratogenic, based on information provided in literature and public databases. Chemicals were tested in all three assays and when results were combined and based on a "2 out of 3 rule", sensitivity and specificity were 96.0% and 71.4%, respectively. The specificity of the devTox assay was increased by incorporating human plasma concentrations into the assay interpretation. When all chemicals were assessed in a decision tree approach, there was an excellent predictivity and assay robustness of 90%. The latter is an interesting predictive approach for evaluating the teratogenic potential of new chemicals.

O-10b-2
Validation and application of NAMs for developmental toxicity testing to predict human safety
ABSTRACT #17

Amer Jamalpoor[1], Sabine Hartvelt1, Jing Fang1, José M. Horcas-Nieto1
1*Toxys B.V., Leiden BioScience Park, The Netherlands*

New approach methodologies (NAMs) offer a valuable opportunity to fill in critical data gaps within chemicals/pharmaceuticals safety assessment, while supporting the 3Rs in the context of developmental and reproductive toxicity (DART) testing. Despite the advancements observed in the past 10 years, confidence in NAMs is still hindered by the limited understanding in their performance and relevance. The ICH S5 (R3) Guidelines on detection of DART are focused on the enhancement of the acceptance of NAMs by streamlining validation requirements and building regulatory trust. Here we present ReproTracker, an in vitro human-based model that has been extensively validated according to ICH S5 guidelines and showcase examples of how it has been incorporated into the DART testing paradigms of the chemical and pharmaceutical sectors. The ReproTracker assay has been employed in battery approaches within multiple DART frameworks—for example, by cosmetic industries to replace animal models in cosmetic testing, and by pharmaceutical and agrochemical industries to either reduce R&D costs and animal use or to provide weight-of-evidence (WoE) data for drugs known to induce teratogenicity based on their mode of action. The combined use of ReproTracker with other NAMs, such as zebrafish or hiPSC-based assays (e.g devTOX quickPredict), enables a more comprehensive understanding of the potential teratogenic effects of compounds. Furthermore, ReproTracker has been evaluated in conjunction with physiologically based pharmacokinetic (PBPK) modeling for several developmental toxicants to predict Human Equivalent Doses (HEDs). These results demonstrate that ReproTracker is a powerful tool within WoE approaches across various DART frameworks, capable of assessing embryo–fetal developmental toxicity.

It holds strong potential to reduce animal use and increase human relevance.

O-10b-3

Application of NAMs in a regulatory context: the pursuit of confidence
ABSTRACT #18

Andy Gibbs[1], Lindsey Parkinson[1]
[1]*Labcorp Inc*

Set against a backdrop of bold political statements, the rapid development and use of NAMS in the area of pharmaceutical development is a “tale of two halves”. On the one hand, use in candidate design, selection, screening and mechanistic investigation is progressing strongly and uptake is becoming more and more widespread. The application of NAMs as a true alternative to in vivo animal models to provide pivotal data in formal regulatory submissions for approval of human clinical trials is still at a stage of early development and utility. This presentation will provide some context on current regulatory expectations in different regions and the climate around the use of data from NAMs in these more formal arenas.

Thursday, 2 July 2026
09:00 - 11:00

Session 11a: Advances & Challenges in High Throughput Transcriptomics for Risk Assessment

Chairs: *Andrew White (Unilever R&D) & Luiz Ladeira (University of Liège)*

O-11a-1

Building confidence in high throughput transcriptomics for chemical safety screening: A framework for assessing reliability and reproducibility of in vitro models
ABSTRACT #34

Logan J. Everett[1], Jacob Fredenburg[21], Joseph L. Bundy[1], Felix Harris[21], Derik E. Haggard[1], Joshua A. Harrill[1]
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Recent technological advancements and decreasing costs have made it feasible to profile all protein-coding genes across thousands of samples with high-throughput transcriptomics (HTTr), allowing for broad evaluation of many molecular targets, signaling pathways and modes of action in a single assay. Assessing the reliability and reproducibility of in vitro HTTr methods for screening chemical bioactivity is critical to their utility and adoption in regulatory applications. We propose a framework to evaluate key outputs of HTTr screening, including 1) estimating the false positive rate for identifying concentration-responsive features by randomly sampling HTTr profiles of cells treated with relatively low concentrations of chemicals to simulate inactive chemical profiles; and 2) estimating the reproducibility of overall transcriptomic points of departure (tPODs) based on repeated testing of several standard reference chemicals throughout large-scale HTTr screening studies. While the individual gene measurements in transcriptomics assays may have lower signal-to-noise compared to targeted assays, our analyses have shown that leveraging coordinated changes across biologically-related gene sets (e.g. pathways) often yields lower false positives and more reproducible tPOD values, compared to concentration-response modeling of individual genes. Advanced analysis methods are also needed to link gene expression changes to relevant molecular targets to predict organism-level hazards. We have developed and systematically compared several novel methods to predict primary molecular initiating events based on HTTr data. Overall, our work has shown that HTTr profiling of chemically exposed cell lines provides a scalable and reliable toxicological screening method with potential utility to multiple use cases and regulatory contexts. Our results can inform best practices and further optimization of methods for transcriptomic concentration-response modeling, which will help build confidence in these assays for use in regulatory decision-making. This abstract does not necessarily reflect US EPA policy. Company or product names do not constitute endorsement by US EPA.

O-11a-2

From research to regulatory application of omics technologies: a broker's perspective bringing researchers, industry, and regulatory bodies together
ABSTRACT #36

Julia M. Malinowska[1], Maurice Whelan[1]
[1]*European Commission, Joint Research Centre*

The European Commission - Joint Research Centre provides independent scientific advice to inform European Union policy, including the development, validation, and application of non-animal approaches in regulatory toxicology. Increasingly, such approaches incorporate complex measurement technologies such as omics (e.g., transcriptomics, proteomics and metabolomics), yet their use in regulatory toxicology remains scarce. Test guidelines of the Organisation for Economic Co-operation and Development (OECD) are considered the pinnacle of international standards for generation of toxicological hazard data on chemicals. The path to including omics technologies within these documents is a challenging one, so how could stakeholders better support inclusion of omics technologies within the future test guidelines? This presentation will focus on use cases and benefits of embedding standards for omics technologies into future test guidelines, which are typically underappreciated. Standards can be both documentary or physical (i.e., reference materials) addressing various aspects of omics-based in vitro workflow: 1) experimental design, 2) exposure of the test system to a chemical, 3) sample collection, 4) sample preparation, 5) data generation, 6) data processing and analysis, 7) data interpretation, and 8) reporting. Incorporating standards within these steps is imperative for adhering to fundamental principles of test guidelines: reliability, accessibility across jurisdictions, and sustainability. The presentation will also discuss the current standardisation landscape of omics technologies (including notable gaps), whilst highlighting both advantages for using standards and opportunities for the stakeholders to accelerate uptake of omics-based in vitro methods in regulatory toxicology.

O-11a-3

Advancement of mechanistic interpretation of HTTr data: an update on the EFSA TXG-MAP project
ABSTRACT #85

Giulia Callegaro[1], Lucy Sinke[1], Sibel Bahtiri[1], Steven Kunnen[1], Imke Bruns[1], Mick van Schaick[1], Julian Suess[2], Marvin Martens[3], Sandra Czech[4], Anne van der Does[5], Marcel Leist[2], Jesper Kers[65], Bob van de Water[1]
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In the context of a safety testing paradigm shift to NAMs, toxicogenomics represents the ideal platform to provide detailed insights into the mechanism of action of chemicals and cover various pathways leading to adverse outcomes (AOPs). However a rigorous and reproducible mechanistic understanding of the several in vitro models available coupled with confidence on the connection between (in vitro) transcriptomic changes and human pathophysiology are still limiting factors to ensure acceptance and wide spread of toxicogenomics. The EFSA funded TXG-MAP project (2023-2027) provides critical components to advance toxicogenomics acceptance for risk assessment. The project supports an unprecedented effort to characterize more than 15 different test systems designed to represent four tissues target of toxicities (liver, kidney, lung and neuronal systems). The test systems range from high-throughput to highly complex systems, including 2D to iPSC derived as well as 3D models. The test systems are characterized for their reproducibility and target tissue representation at the gene level. Gene co-expression networks are constructed for each model separately from a large perturbational dataset and advanced interpretation framework are applied, including mapping to the AOP framework. Additionally, we collect and analyse large human biopsies collections, enabling the construction of

human- and adverse-related co-expression models. Test systems are compared to each other and to human biopsies at the network level, to determine their differential predictivity towards human pathogenesis. Data and models are part of the TXG-MAPr platform (https://txg-mapr.eu/login/), a shiny-based interactive tool designed to aid utilization of network models to a larger audience and enhance HTTr interpretation in toxicology practice. This work was supported by the European Commission's Horizon 2020 program RISK-HUNT3R project (GN 964537), and carried out in the framework of the European Partnership for the Assessment of Risks from Chemicals (PARC) (GA 101057014). This works is currently supported by EFSA (TXG-MAP).

O-11a-4

Learning from industry case studies: addressing the challenges in reliability and uncertainty in the implementation of toxicogenomics
ABSTRACT #91

Andrew White[1], Joe Reynolds[1], Sophie Cable[1], Maria Teresa Baltazar[1], Jade Houghton[1], Sophie Malcomber[1], Claire Peart[1], Alistair Middleton[1], Georgia Reynolds[1], Sharon Scott[1], Predrag Kukic[1]
[1]*Unilever R&D*

High throughput transcriptomics (HTTr) has emerged as a central tool in next-generation chemical risk assessment. Such NAM based tools are now being assessed as part of regulatory agencies transition plans to increase the use of NAMs highlighting the transition within their future work plans such as EPA, EFSA and the development of the EU roadmap towards phasing out animal testing. By characterising changes in in vitro gene expression levels with increasing chemical concentrations, HTTr can provides both insights into the molecular mechanisms underlying chemical toxicity alongside determining bioactivity-based points of departure (tPOD). The falling cost of this technology is allowing the rapid dose response screening of numerous chemicals nevertheless its implementation in regulatory decision making and frameworks still faces several critical challenges. For example, a lack of standardisation of protocols and data analysis methods impacting the reproducibility and variance in findings. This talk will explore recent work from several exposure led case studies to understand the utility and limitations of HTTr data as an untargeted screening tool within a NGRA toolbox for complex endpoints such as systemic toxicity. Using core characteristics of scientific confidence and readiness frameworks we assessed the uncertainties supporting the decisions for the case studies and how addressing them has strengthened the credibility of the interpretation especially in relation to the internal exposure estimates from pharmacologically based kinetic models. This will include assessment of the robustness and reproducibility of the generated data, approaches for interpretation and analysis including updates on the application of the Bayesian Bifrost statistical model, and the use of workflow management tools to drive consistency, scalability, portability and transparency in the tPODs determined. In this manner we highlight how these qualification approaches can support the acceptance of such omic based NAMs within a broader NGRA toolbox.

O-11a-5

ICCS activities in the application of toxicogenomics for cosmetic safety science. A universal intuitive tool for toxicogenomics applications
ABSTRACT #92

David Allen[1]
[1]*ICCS*

Many applications for toxicogenomics data have been proposed for use in safety assessment. This includes grouping and read across, point of departure calculations, and mode of action (MoA) determination. For toxicogenomics data analyses and their application in safety assessment to be accepted in the regulatory framework they must be robust, scientifically valid, and fully documented. However, there is a paucity of user-friendly tools that can accomplish these tasks efficiently and effectively. The International Collaboration on Cosmetics Safety (ICCS) helps align innovative scientific approaches with evolving regulatory expectations, helping to protect safe

ingredients and enable innovation. Accordingly, ICCS is developing a computational tool to facilitate user friendly applications of grouping and read across to make these analyses accessible to those without in depth technical skills. The tool comprises a detailed workflow for grouping and read across and contains place holders for POD calculations and MoA determination, which will be added to future iterations. We considered connectivity mapping (CMap) to be the most well-developed technology and thus formed the basis of the workflow for the tool. The tool supports a consistent process for transforming data into transcriptomic profiles, provides a convenient frontend for the OECD Omics Reporting Framework, and integrates modern analyses by proven providers. The inherent scientifically robust, universally accessible, and transparent nature of the tool should aid in establishing confidence in toxicogenomics methods as part of a regulatory framework. ICCS intends to make this tool open source, and as such the system architecture is meant to be accessible to a multitude of environments and deployment strategies.

Session 11b: Guidance for In Vitro Air-Liquid interface Inhalation Toxicity Assessment

Chairs: *Tanja Hansen (Fraunhofer ITEM, Germany) & Andreas Stucki (PETA Science Consortium International e.V.)*

O-11b-1

In-vitro toolbox for predicting inhalation toxicity in humans
ABSTRACT #58

Tanja Hansen[1], Detlef Ritter[1], Katharina Schwarz[1], Abishek Laxmanan Ravi Shankar[1], Christina Drake[1], Rayane Boufalaas[2], Pierre-Andre Billat[2], Catherine Gabriel[3], Sylvia Escher[1]
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The air-liquid interface (ALI) culture technique represents the state-of-the-art method for in vitro lung-related research, enabling exposure to aerosols and gases (1, 2). This approach, utilizing A549 cells, has demonstrated a promising correlation between in vitro and in vivo results. The significance of this testing setup has been confirmed for gases, volatile chemicals, and fungicides, which were applied as dry powder aerosols. A continuous flow exposure system was employed to ensure controlled and highly relevant exposure of ALI cultures, as well as precise dosimetry. Successful aerosol exposure also requires additional technical measures to enable the deposition of effective exposure doses in particle size ranges relevant for inhalation into the deep lung (200 – 800 nm), which was achieved through thermophoresis. Cytotoxicity was assessed using the WST assay, and dose-response curves were established. The EC50 values obtained from in vitro testing were correlated with LD50 values from acute rat inhalation in vivo studies, demonstrating strong predictivity. A549 cells exhibited predictive responses to both hydrophilic (e.g., tert-butyl hydroperoxide, isobutanol) and hydrophobic volatiles (e.g., styrene, toluene), as well as to dry powder aerosols. To estimate the extent of systemic absorption upon inhalation, quantification of pulmonary uptake is essential. Accordingly, apparent permeability coefficients (Papp) were determined using human lung barrier models (Calu-3, hAELVi) and incorporated as input parameters for our inhalation PBK model, PBKiT. Examples of in vitro to in vivo correlations will be presented. In conclusion, our findings indicate that a combined approach utilizing in vitro data alongside in silico modeling can effectively predict acute local lung toxicity and the bioavailable dose following inhalation exposure in humans.

O-11b-2

Harmonizing lung cell models: insights from inter-laboratory comparison studies
ABSTRACT #62

Barbara Rothen-Rutishauser[1]
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Inhalation toxicology is a field of intensive research for hazard and risk assessments of pharmaceutical and environmental aerosols. Regulatory toxicology is still mainly based on

animal testing; however, a range of various 3D lung models have been established over the last few years to contribute to a mechanistic understanding of the aerosol and can contribute to dose selection. Despite this significant progress in increasing complexity and physiological relevance, the models are still restricted for use in research environments. For the broader acceptance of in vitro lung models, robustness, reproducibility, and predictivity must be demonstrated [1]. Several approaches and efforts are currently ongoing to close these gaps, such as projects that address standardization by testing the transferability and reproducibility of in vitro lung cell systems via interlaboratory comparison studies [2, 3]. This presentation will cover the challenges of protocol harmonization and the information required to perform an alternative test, including an itemized list of employed materials and devices, detailed instructions on experimental procedures to follow, positive and negative controls, as well as performance criteria. Such detailed information is key to harmonizing the testing and to confirm reproducibility and quality, as well as comparability of data and results from the same method between different projects (different personnel involved) and in different laboratories. Case studies will be presented to demonstrate how harmonized protocols can provide comparable results among different laboratories. This is key to increasing confidence in approaches to testing substances for respiratory and inhalation toxicity that do not require the use of animals.

O-11b-3

Standardization of cell air-liquid interface studies to assess the toxicity of air pollutants
ABSTRACT #67

Camille Bonnefous[12], Louise Verhaeghe[12], Suen Boulé[12], Dominique Courcot[12], Frédéric Ledoux[12], Yann Landkocz[12]
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Air pollution, caused by particulate matter and gases from natural and anthropogenic sources, contributes to numerous premature deaths each year. Fine particles, nitrogen dioxide, and ozone are well-established threats [1]. Emerging pollutants —such as micro- and nanoplastics, ultrafine particles, and VOCs like 1,3-butadiene or hydrogen sulfide—are now under increasing investigation. Air pollution is linked to respiratory and cardiovascular diseases including asthma, fibrosis, and chronic obstructive pulmonary disease. In 2013, the IARC classified outdoor air pollution and particulate matter as carcinogenic [2]. Since the respiratory system is the primary entry point for airborne pollutants, air-liquid interface (ALI) models have been developed to better mimic lung physiology in toxicity studies. We have established a standardized ALI methodology for assessing air pollutants, either under controlled lab conditions or directly in the field. Initially, we characterize the cell types used, checking for markers of alveolar Type I and II cells, as well as basal, ciliated, and secretory epithelial cells. We then evaluate several endpoints: epithelial barrier integrity (trans-epithelial electrical resistance measurements and paracellular permeability), cellular viability and proliferation (ATP, LDH, lactate, AlamarBlue), mucus production, and cytokine release (ELISA). We also assess xenobiotic metabolism and oxidative stress. To support mechanistic insights, we integrate -omics data such as RNA sequencing. In parallel, pollutant metrology is systematically performed to quantify exposure doses. This approach was deployed during outdoor air exposure campaigns (exposure of the general population), in working conditions (toxicity of wood dust in carpenters) or during controlled studies (exposure scenarios encountered in the field). Future inter-laboratory validation of our protocol may enable harmonization of ALI practices. This would promote a human-relevant, mechanistically informative strategy for inhalation toxicity assessment aligned with modern regulatory and scientific expectations.

O-11b-4

Advancing respiratory tract cell culture NAM models for risk-based decision making
ABSTRACT #26

Jo Wallace[1], Mary McElroy[1], Kalyani Guntur[2], Mitch Klausner[2], Seyoum Ayehunie[2]
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Toxicity of a panel of 10 chemicals known for point-of-contact toxicity in the lung was evaluated in primary human bronchial epithelial cells (2D) and 3D human and rat EpiAirway™ tissue models via direct liquid application to the epithelial surface following an acute (4h) exposure and 20h recovery. Experiments were conducted at Charles River UK and MatTek USA using a harmonized protocol. Barrier integrity (TEER), cell viability (Alamar Blue), morphology changes (H&E staining) using A.I. analysis algorithms (Visiopharm), changes in gene expression and cytokine release were used as endpoint measurements. Statistical analysis was performed comparing interlaboratory comparisons and calculation of EC10/25/50 parameters. Toxicity measurement showed high reproducibility between labs with 9/10 (2D) and all (3D) chemicals meeting criteria. Monolayers were more sensitive than 3D-human tissues for 9/10 chemicals, varying by chemical. All models reliably ranked materials for hazard. Human EpiAirway EC10 values <5mg/mL predicted toxic materials (Categories 1-3), while values >highest concentration feasible indicated Category 5. Morphological changes corresponded with toxicity data in the 3D-human model. The 3D-rat model showed comparable reproducibility, and generally aligned with the 3D-human results, although with slight species differences. Inflammatory markers showed model and chemical specific differences in response. Changes in gene expression were also assessed. The data gathered so far show that harmonized protocols facilitate inter-laboratory reproducibility. This tiered approach shows potential for predicting human inhalation hazard classifications and supports the development of an IATA. This work is part of a larger program including repeat dosing, aerosol application, and donor-to-donor variability, with the eventual goal of generating a prediction model. Evaluation of the in vitro toxicological platforms for aerosol chemical screening aligns with the regulatory strategic goal 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.

O-11b-5

Impact of in vitro dosimetry on IVIVE and risk assessment. ABSTRACT #29

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In vitro particle dosimetry is essential for accurately assessing the toxicological impacts of inhaled particulate matter on respiratory tract and lung epithelial cells and macrophages. Traditional submerged culture models, while widely used, present challenges in simulating realistic exposure scenarios due to the influence of sedimentation, diffusion, and agglomeration dynamics that affect particle delivery to the cell surface. Consequently, understanding and quantifying the actual dose delivered to cells, rather than relying on nominal concentrations, is critical for meaningful interpretation of biological responses. In contrast, air-liquid interface (ALI) cultures offer a more physiologically relevant platform, particularly when mimicking aerosol exposure conditions encountered in vivo. Continuous flow exposure systems, such as aerosol-to-cell deposition devices, have emerged as advanced tools to expose ALI cultures to airborne particles in a controlled and dynamic manner. These systems require precise dosimetry models to capture deposition efficiency, temporal dose profiles, and the physicochemical transformations of particles during transport. Accurate dosimetry across both submerged and ALI conditions enables better cross-comparison of experimental data and facilitates dose-response modeling, which is vital for hazard assessment and regulatory decision-making. Furthermore, integrating dosimetry into experimental design improves the relevance of in vitro models for risk assessment by bridging the gap between laboratory studies and real-world exposures. As nanomaterials (advanced materials) and complex aerosols become more prevalent, refining dosimetry methodologies in continuous flow exposure systems is imperative to advance inhalation toxicology and to support the development of safer materials and more

effective exposure regulations. This presentation will highlight the need for proper characterization of the exposures using in vitro models.

O-11b-6

Adverse outcome pathways (AOPs) and selection of biomarkers
ABSTRACT #41

Ulla Vogel[12], Claudia Gutierrez[2], Jorid Sorli[1], Sabina Halappanavar[3]
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Adverse outcome pathways (AOP) are structured representations of biological events leading to adverse effects and are considered relevant to risk assessment by the OECD. Existing AOPs can be found at https://aopwiki.org/. The AOP links in a linear way existing knowledge along one or more series of causally connected key events (KE) between two points — a molecular initiating event (MIE) and an adverse outcome (AO) that occur at a level of biological organization relevant to risk assessment. The linkage between the events is described by key event relationships (KER) that describe the causal relationships between the key events. One major advantage of the AOP concept is that an AOP creates a causal link between early KEs that may be assessed in vitro and adverse outcomes. Quantitative AOPs (qAOP) include information on dose-response relationships and may be used in regulation for derivation of health-based limit values. Pulmonary exposure to particles and chemicals may result in adverse health effects in the lungs and in secondary organs including lung cancer, fibrosis, emphysema, and cardiovascular disease (1). In this presentation, examples of AOPs of relevance to inhalation exposure with focus on inhalation of particles. The first example is the OECD-endorsed AOP273 describing how inhalation exposure to substances may lead to fibrosis (2). The second example is AOP237 describing how inhalation exposure may lead to atherosclerosis (3). Examples of KEs and relevant biomarkers as well as dose-response relationships for quantitative AOPs will be presented.

Thursday, 2 July 2026
11:30 - 12:45

Session 12a: AI-Powered Toxicity Screening: Enhancing In Vitro Models, 3Rs, and Regulatory Adoption.

Chairs: *Jian Jiang (Vrije Universiteit Brussel, Belgium) & Jinhee Choi (University of Seoul)*

O-12a-1

Evidence-based AI: from trailblazer to trustblazer?
ABSTRACT #493

Thomas Hartung[12]
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Agentic AI systems can plan, call tools, and coordinate specialized sub-agents, enabling multi-step scientific workflows that exceed what single-model text generation can reliably deliver. Yet in high-stakes domains such as regulatory science and toxicology, fluent outputs are not sufficient: adoption hinges on traceability, reproducibility, context-of-use validity, and explicit uncertainty communication. This perspective argues that evidence-based medicine and evidence-based toxicology provide a mature epistemic scaffold for making agentic AI trustworthy by design. We propose an Evidence-based Agent Stack that decomposes end-to-end tasks into protocolized roles (question framing, retrieval, screening, extraction, risk-of-bias appraisal, synthesis, mechanistic/causal integration, uncertainty assessment, and evidence-to-decision translation) with mandatory provenance and versioning. Anchoring agentic workflows in systematic review practice, risk-of-bias frameworks, and emerging regulatory principles (e.g.,

TREAT and e-validation) can turn "trailblazing" AI into "trustblazing" AI: systems whose outputs are auditable, ~~updateable, and aligned with decision~~ accountability.

O-12a-2

Practical applications of language models in the toxicological context. ABSTRACT #42

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Will be provided Toxicology is shifting from animal test–centric assessment toward human-relevant, mechanistic science grounded in New-Animal Methodologies (NAMs), Next-Generation Risk Assessment (NGRA), and the Adverse Outcome Pathway (AOP) framework. While AOPs structure biology across key events and their (causal) relationships, the scale and heterogeneity of evidence—and the need for provenance—remain major bottlenecks. This talk outlines how large language models (LLMs) and agentic workflows can accelerate evidence retrieval and synthesis without replacing expert judgement. We demonstrate: (i) retrieval-augmented generation (RAG) grounded in authoritative sources (e.g., OECD AOP-Wiki, PubMed, ToxCast/Tox21); (ii) chemical/biomedical entity normalization and causal relation extraction to support KE/KER curation and read-across; (iii) AI agents that query knowledge graphs to assemble candidate AOP subnetworks and highlight data gaps; and (iv) workflow patterns that expose uncertainty, track citations, and produce reproducible, FAIR evidence reports. Case studies on liver, thyroid, and kidney toxicity show how these tools prioritize hypotheses, triage studies, and better connect mechanistic data to NGRA decision contexts. Because LLMs can overgeneralize and confabulate, we emphasize safeguards essential for deployment and use: strict grounding and provenance, tool-augmented reasoning and task-specific evaluation, such as screening and triage, and human-in-the-loop workflows) Furthermore, we strive for alignment with emerging governance, e.g. the EU AI Act transparency and documentation duties for general-purpose AI. We also share lessons from focused hackathons that aim to rapidly prototype AI models and applications and publish reusable artifacts for the community, lowering the barrier to adoption and encouraging benchmarking. Take-home message: With appropriate guardrails, LLMs plus agents can make mechanistic toxicology faster, more transparent, and more reproducible—freeing experts to focus on judgement and decision-making rather than document wrangling.

O-12a-3

A neuro-symbolic AI approach for predicting blood–brain barrier penetration to screen for potential neurotoxicants ABSTRACT #31

Swapnil Chavan[1]
[1]*Research Institutes of Sweden RISE*

An adverse outcome pathway (AOP) maps the relationship between a stressor-induced molecular initiating event (MIE) and an adverse outcome (AO) through key events (KEs). For some AOPs, such as neurotoxicity, access to the target site, often through the blood-brain barrier (BBB) can be rate-limited. In quantitative AOPs (qAOPs), understanding the quantitative relationships between MIEs, KEs, and the factors influencing them is crucial. This study explores whether crossing the BBB is rate-limiting in neurotoxicity adversity and investigates if the amount of a chemical stressor crossing the BBB can serve as a quantitative MIE. We introduce a novel multi-task deep learning approach that combines symbolic AI (rules) with statistical AI to predict both BBB penetration as a discrete class and the logBB (CSF/plasma concentration ratio) values. By explicitly representing domain knowledge, including known pharmacological principles, structural features, and physico-chemical properties, we incorporate ten different structural rules to apply logical constraints during model training. This work demonstrates that combining symbolic reasoning with deep learning techniques can

provide a powerful tool for both qualitative and quantitative BBB penetration and may serve as a valuable tool towards predicting neurotoxicity.

O-12a-4

AI-powered virtual control groups: reducing animal use in preclinical safety assessment
ABSTRACT #56

Julia Matyjasiak[1], Paolo Piraino[2], Marta Juncarol[3], Barbara Fuzi[3], Mathieu Vinken[1], Jian Jiang[1]
[1]*Vrije Universiteit Brussel, Brussels, Belgium*
[2]*Organon SRL, Bucharest, Romania*
[3]*Barcelona Supercomputing Center, Barcelona, Spain*

Artificial intelligence (AI) is transforming safety assessment by driving innovation in New Approach Methodologies (NAMs) and advancing the 3Rs (Reduction, Replacement and Refinement) in toxicology research. The VICT3R project (Developing and implementing VIrtual Control groups To reducE animal use in toxicology Research) exemplifies this shift by leveraging historical control data (HCD) to develop AI-powered Virtual Control Groups (VCGs) that reduce animal use while maintaining scientific and regulatory standards. Thousands of HCD records from diverse studies were collected from VICT3R consortium partners to support synthetic data generation and modeling. This valuable resource strengthens preclinical safety assessments and accelerates NAM development and adoption. As a major challenge, the variability of HCD often causes data sparseness. AI and machine learning (ML) are powerful tools to increase dataset completeness by generating missing variables. In this study, we assumed that animal growth can be leveraged to optimise imputation and explored growth curves – within-animal changes in body weight (BW) over time – and their association with several other common study endpoints. BW data from Sprague Dawley and Wistar Han rats in the VICT3R database were fitted using established rat growth models (such as log-linear, Gompertz). Model parameters were extracted and correlated with common toxicological variables (organ weights, clinical chemistry markers, hematological outcomes). For predicting age, we used BW data in both simple models (inverted spline fits) and ML approaches (random forests, Multiple Imputation by Chained Equations). For correlated toxicological parameters, we evaluated whether growth curve-derived parameters outperform raw BW values. Imputation performance of these inputs was compared using ML methods (random forests, gradient boosting) and a generative AI (Synthetic Data Vault framework). Preliminary findings indicate that the BW trends together with AI outperforms other statistical approaches in imputation of age and other endpoints, supporting AI-enhanced VCG development in cases of incomplete historical data.

Session 12b: The application and standardisation of in vitro inhalation toxicology methods for academia and industry

Chairs: *Arno Gutleb (Invitrolize, Luxembourg) & Ian Crooks (British American Tobacco)*

O-12b-1

Precision-cut lung slices provide an immune-responsive, long-term respiratory model for complex adverse event detection that is amenable for standardized research
ABSTRACT #7

Holger Behrsing[12], Vivek Patel[1], Ramaswamy Krishnan[3], Bela Suki[43], Sangita Choudhury[567], Sylvia Balbo[8], Stephen Hecht[8], Khalid Amin[8]
[1]*Institute for In Vitro Sciences, Inc.*
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[8]*University of Minnesota*

Scientists are increasingly exploring new approach methodologies (NAM) to identify dependable and accurate non-animal alternatives for predicting human toxicities. Several three-dimensional (3D) test systems have emerged as excellent models of the human respiratory tract. Among these, precision-cut lung slices (PCLS) provide a highly relevant model because they contain

many cell types, including immune cells and small airways, and preserve the native respiratory architecture of the deep lung. Traditionally, PCLS were often cultured using a submerged or roller culture method, but this approach prevented their use in aerosol exposure systems designed for tissue culture inserts. Recent work demonstrates that PCLS thrive in an air-liquid interface (ALI) environment, and scientists now use them more frequently for aerosol exposures. While a lack of long-term PCLS preservation previously limited the PCLS model's use in repeat-donor and mainstream testing, recent advances in cryopreservation now allow donor banking and PCLS experimentation within days of thaw. Both fresh and frozen-thawed PCLS retain key cell types and demonstrate equivalent functional response for over 1 month in culture. We will review the application of hPCLS as a model of the lung, showing short- and long-term exposure responses. These include pro-drug (metabolically activated) triggered acute cytotoxicity, silica-induced inflammation, pro-fibrotic driven ECM deposition, reference cigarette aerosol exposure-induced DNA adduct formation and more adverse event (AE) responses that followed media-based ("systemic") or aerosol-based ("inhalation-like") exposures to PCLS at ALI. Recent work suggests spatial transcriptomic measurements validate the applied cryopreserved-thawed PCLS as a biologically accurate and spatially informative model when interrogating differences between normal and diseased tissues. The ability to access banked PCLS "on-demand" presents this sophisticated 3D test system as a tool for detection of AE using repeat- and long-term exposure. Applying cutting-edge endpoint detection further positions PCLS as a NAM option for screening and sophisticated evaluation of exposure-induced pulmonary tissue responses.

O-12b-2

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
ABSTRACT #74

Michael Hollings[1]
[1]*Labcorp*

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 (continuous flow versus static). 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 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 analysed for nicotine 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-disulfophenyl)-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 24hr 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.

O-12b-3

Adverse outcome pathway (AOP)-based in vitro evaluation of mucus hypersecretion for comparative toxicological study of combustible cigarette smoke and heated tobacco product aerosols

ABSTRACT #14

Shigeaki Ito[1], Sakuya Ichikawa[1], Risa Matsumoto[1], Kazuo Erami[1]
[1]*Japan Tobacco Inc.*

The adverse outcome pathway (AOP) framework is an analytical strategy that facilitates more effective and simplified toxicological assessments. An AOP consists of a molecular initiating event (MIE), key events (KEs), and an adverse outcome (AO), representing the causal sequence leading to the AO. In this study, we applied the AOP framework to inhalation toxicology using 3D-cultured in vitro airway epithelial cells. The AOP starts with oxidative stress as the MIE, proceeds through intermediate KEs, including goblet cell metaplasia/hyperplasia (GCM/H), and culminates in mucus hypersecretion as the AO. Because mucus hypersecretion is a hallmark symptom in chronic airway diseases, its evaluation provides valuable insights into how inhaled substances influence the risk of disease progression. We conducted a comparative study between combustible cigarette smoke and heated tobacco product (HTP) aerosol. HTPs are considered potential reduced-risk products due to their aerosols containing lower concentrations of harmful constituents than cigarette smoke. Whole aerosols from both products were exposed to 3D airway epithelial cells from six different donors, six times each. At each exposure, data on MIE, KEs, and AO were collected to assess time-dependent biological changes. Despite donor variability, cigarette smoke consistently induced all biological events in the AOP, including tissue phenotype changes (GCM/H) and mucus hypersecretion (AO). As expected, later KEs and AO increased with repeated exposure, reflecting the risk of disease progression. In contrast, HTP aerosol elicited much less pronounced effects, especially for later KEs and AO, even at higher concentrations than cigarette smoke. These results suggest that HTPs have the potential to reduce health risks associated with mucus hypersecretion. This presentation will also include discussion of deposition modeling-based exposure assessment, aiming to better relate actual user inhalation scenarios to the in vitro exposure conditions employed in this study.

O-12b-4

Development of the in vitro micronucleus test in human-derived 3D EpiAirway tissues for exposure to aerosols from cigarettes, HTP and vapour products

ABSTRACT #70

Ian Crooks[1], Michael Hollings[2], Robert Bedford[2], Damien Breheny[1], Emma Bishop[1]
[1]*B.A.T. (Investments) Ltd.*
[2]*Labcorp*

3D skin models can be used to assess genotoxicity with the in vitro micronucleus test (IVMN), while organotypic lung models are underdeveloped for genotoxicity endpoints. The genotoxicity of inhaled substances could be investigated in 3D lung tissues, adding a more biologically relevant exposure and relevant tissue model, which could complement standard cell culture methods. This study developed the IVMN in 3D EpiAirway tissues and investigated its potential to assess comparative genotoxicity of cigarettes, Heated Tobacco Products (HTPs) and electronic nicotine delivery systems (ENDS). EpiAirway tissues were exposed to positive controls MMC and vinblastine. Aerosols from the 1R6F research cigarette, an HTP and ENDS were generated on Vitrocell equipment, diluted with humidified air and delivered to the surface of the EpiAirway tissues. 1R6F smoke was exposed for up to 1 hour and the HTP and ENDS aerosols exposed for up to 2 hours. Nicotine deposition was measured to confirm aerosol exposure. Tissues were allowed to recover for 48 hours, with the addition of epidermal growth factor (EGF) to enhance cell turnover. Micronuclei induction was determined using Litron MicroFlow kits. MMC and vinblastine induced dose-related increases in micronuclei, demonstrating validity of this model. 1R6F smoke induced dose-related increases in micronuclei. The HTP aerosol induced a weak micronucleus response of no biological relevance and significantly less than

1R6F. The ENDS did not induce micronuclei, despite testing with a more concentrated aerosol over a longer period than for the 1R6F. The results indicate that it is feasible to obtain a genotoxic response from positive controls and 1R6F smoke. However, further investigation is required into additional positive controls and understanding the metabolic capacity of the tissue for pro-genotoxicant exposure.

O-12b-5

Implementing GIVIMP: A practical pathway toward quality and compliance in in vitro toxicology ABSTRACT #68

Sabina Burla[1], Amanda Ulrey[2], Arno C. Gutleb[1]
[1]*Invitrolize*
[2]*IIVS*

With growing reliance on in vitro approaches for chemical safety evaluation, ensuring data integrity and reproducibility has become essential. The OECD's 2018 Guidance Document on Good In vitro Method Practices (GIVIMP) provides a structured framework to support the development and regulatory acceptance of alternative test methods, particularly in contexts where reproducibility and transparency are vital. Beginning in early 2024, a GIVIMP implementation initiative was undertaken with the support of an external auditing organization. The approach to implement GIVIMP includes compiling extensive documentation - from facility blueprints and procurement processes to standard operating procedures and data management protocols. A two-day on-site audit assesses operations, and is followed by a gap analysis report. Identified deficiencies are addressed through refined reporting routines, improved documentation, and strengthened quality assurance practices. After formal accreditation has been achieved, the adoption of GIVIMP principles immediately can lead to more robust workflows, clearer documentation within the organisation, and enhanced credibility in the eyes of stakeholders. Early feedback from partners suggests that visible commitment to quality standards strengthens trust and supports the method's broader regulatory acceptance. Adopting GIVIMP from the outset of in vitro method development, regardless of organizational size or maturity, delivers significant advantages. Establishing a culture of quality early simplifies later scaling, ensures staff adherence to best practices, and can accelerate the path toward regulatory recognition. These findings highlight the importance of treating GIVIMP as the default standard for modern in vitro toxicology.

Tuesday, 30 June 2026
16:30 - 17:30

Session ESR1: Early Stage Researcher Session 1

Chairs: *Rita Ortega-Vallbona (ProtoQSar) & Luiz Ladeira (University of Liège)*

O-ESR1-1

Establishment of an in-vitro neurotoxicity test battery using 2D/3D neuronal cell models for the assessment of functional disturbances in the nervous system ABSTRACT #109

Eike Cöllen[1], Jasmin Kormann[2], Yaroslav Tanaskov[1], Chiara Wolfbeisz[1], Anna-Katharina Holzer[1], Udo Kraushaar[2], Marcel Leist[13]
[1]*University of Konstanz*
[2]*NMI Natural and Medical Sciences Institute at the University of Tuebingen*
[3]*CAAT-Europe, University of Konstanz*

Neurotoxicity in adults (NT) and during development (DNT) is a major safety concern for all chemicals of the human exposome. Exposure to chemicals has been linked to the increasing prevalence of some neurodegenerative diseases and autism spectrum disorders. Currently available tests, such as the DNT in-vitro battery (DNT-IVB) address only some functional alterations in key neurodevelopmental processes (KNDP) or neuronal network function. We aimed at addressing remaining gaps. Moreover, we wanted to provide tools for a detailed screen hit follow up and toxicological characterization in human cell-based assays. Therefore, we developed several functional neurotoxicity assays to investigate some of the existing gaps in the DNT-IVB EU. These assays address

different levels of complexity of functional neurotoxicity that can assess both the modulation of spontaneous network activity and the response to specific stimuli. The endpoints used are changes of [Ca2+]i, receptor desensitization, changes of receptor expression, alterations of neuronal connectivity, and the toxicant-induced alterations of complex oscillatory activity in neurons. We established these assays for various neuronal cell types like human dopaminergic neurons, or hiPSC-derived peripheral neurons, and hiPSC-derived mixed cortical cultures, as well as glial cells, like hiPSC-derived astrocytes. The functional neurotoxicity battery consists not only of 2D cell culture models but also incorporates 3D neuronal spheroids to increase the biological coverage. With these assays, it is possible to cover a wide range of different chemicals (e.g. neonicotinoids, pyrethroids, strobilurins and methylpyrazole pesticides). The battery identifies chemicals targeting several receptor classes like NMDA, glutamate, nAChR, purinergic receptors, as well as various ion channels like voltage-gated sodium channels (VGSCs). Overall, the first results provide a valuable addition to the original DNT-IVB to cover some of the existing gaps for functional neurotoxicity.

O-ESR1-2

A generalizable approach to establish neurotoxicity assays with heightened sensitivity for mitochondrial toxicants
ABSTRACT #112

Mahshid Alimohammadi[1], Marcel Leist[12], Sarah Kucharczyk[1], Silvia Tangiany[1], Anna-Katharina Holzer[1], Julian Süss[1], Viktoria Magel[1], Eike Cöllen[1], Naja Bürgers[1]
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[2]*Center for Alternatives to Animal Testing in Europe (CAAT-Europe), 78457 Konstanz, Germany*

Background: Conventional in vitro studies often lack sensitivity to mitochondrial toxicants due to the glycolytic bias of cultured cells maintained in high-glucose media. While replacing glucose with galactose can force mitochondrial energy reliance, this modification is not feasible in all cell systems or media formulations. Therefore, we investigated the use of the glucose transporter inhibitor Glutor (GTor) as a universal supplement to shift cellular energy production toward mitochondrial pathways. Methods: The human dopaminergic LUHMES cell-based NeuriTox (UKN4) assay was employed to assess metabolic shifts and toxicity following GTor treatment. Lactate production was measured to monitor glycolysis, and the complex I inhibitor tebufenpyrad (TEBU) was used as a model mitochondrial toxicant. Toxicity was evaluated via neurite area measurements. Broader applicability was tested using other mitochondrial and non-mitochondrial toxicants, as well as in two additional human iPSC-derived neurodevelopmental test systems. Results: GTor induced a concentration- and time-dependent reduction in lactate production, confirming glycolysis inhibition. In the presence of 100 nM GTor, the neurotoxicity of TEBU was enhanced approximately 5000-fold (from BMC25 = 50 µM to 10 nM). Similar sensitization was observed with other complex I inhibitors. The enhanced mitochondrial toxicity detection was replicated across other LUHMES-based assays and in peripheral neuron and neural crest cell models. Conclusions: GTor supplementation effectively shifts cellular metabolism and significantly increases sensitivity to mitochondrial toxicants. This broadly applicable strategy improves the relevance of cell-based neurotoxicity assays and enables robust detection of mitochondrial dysfunction, even in systems where galactose substitution is impractical.

O-ESR1-3

Enhancing mechanism-based neurotoxicity evaluations with in vitro LUHMES-based gene co-expression network analysis
ABSTRACT #113

Lucy Sinke[1], Julian Suess[2], Steven Kunnen[1], Hugo van Kessel[1], Marcel Leist[2], Giulia Callegaro[1], Bob van de Water[1]
[1]*Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.*
[2]*University of Konstanz, Konstanz, Germany.*

Next-generation risk assessment (NGRA) requires the integration of efficient, cost-

effective, and biologically relevant new approach methodologies (NAMs) into safety assessment frameworks. While toxicogenomics has potential to provide valuable mechanistic insights, current implementations primarily focus on individual gene-level changes and generic gene sets. This is despite cellular stress responses and adverse outcome pathways typically involving coordinated regulation of multiple genes in tandem, possibly in tissue- and cell-type specific manners. For liver and kidney test systems, the TXG-MAPr webtool offers an accessible and interactive platform to assess molecular perturbations induced by novel compounds and explore early events leading to toxicity. However, there is thus far no equivalent resource for interrogating neuronal test systems and neurotoxicity endpoints. Here, as part of the EFSA TXG-MAP project, we present the first neuronal TXG-MAPr, based on developing dopaminergic neurons (LUHMES d2-3). Our network is generated on the basis of exposure to 43 relevant compounds, triggering distinct cellular responses representative of specific biological perturbations. Transcriptomic responses were captured across multiple concentrations and exposure times. Using weighted gene co-expression network analysis (WGCNA), we identify functionally meaningful gene modules that capture key neurotoxic and general stress responses. Module annotations include several well-characterized pathways including oxidative and ER stress, alongside neuronally specific ones relating to neurogenesis and neurodegeneration. Moreover, we highlight the utility of the TXG-MAPr tool by projecting an independent external dataset onto the LUHMES-derived network. By exploring the ability of our modules to distinguish between high- and low-toxicity compounds, we showcase the applicability of such methods in early-stage compound assessment and predictive toxicology. Taken together, this work highlights the power of gene co-expression network analysis in a neuronal test system and offers a mechanistic framework for system-specific toxicogenomic evaluations in neurotoxicity research moving forward.

O-ESR1-4

Threshold of toxicological concern (TTC) and new opportunities in the era of AI

ABSTRACT #118

Matthias Wehr[1], Eliska Kuchovska[2], Karolina Kopańska[3], Thomas Hartung[34], Grace Patlewicz[5], Julia M. Malinowska[6], Sylvia Escher[1]

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[6]*European Commission, Joint Research Centre (JRC), Ispra, Italy*

The Threshold of Toxicological Concern (TTC) concept establishes limit values below which the risk to human health is considered negligible. These limit values are derived from the 5th percentile of the distribution of no observed effect levels, a probabilistic approach. Within the Next-Generation Risk Assessment (NGRA) frameworks promoted by the ASPIS cluster, TTC serves as one of the key components for enabling safety evaluations without animal testing in the absence of chemical-specific toxicity data. During the CAAT-ONTOX workshop in 2025, the potential of artificial intelligence (AI) methods to enhance probabilistic risk assessment and the TTC framework was discussed. This presentation will share several proposed ideas to improve the TTC concept, particularly focusing on the improvement of Cramer classes. It was discussed how AI can be applied to i) aggregate and integrate data effectively, and ii) refine the classification of TTC categories using novel methodologies. Additionally, the possibility of establishing NAM-based TTC, category-specific thresholds and incorporating new

endpoints, as well as developing specific thresholds for alternative exposure routes such as inhalation and dermal exposure, will be addressed. The successful implementation of these probabilistic methods necessitates access to extensive, high-quality databases, which may become feasible in the future, considering ongoing initiatives by ECHA and EFSA to promote shared data platforms. Ultimately, integrating AI and probabilistic risk assessment into the TTC and read-across approaches could significantly enhance the robustness and applicability of risk assessments in toxicology.

O-ESR1-5

ONTOX Probabilistic Risk Assessment (OPRA): An AI-enabled framework
ABSTRACT #490

Karolina Kopańska[1], Thomas Hartung[1], Alexandra Maertens[1], Thomas Bozada[2], Eliška Kuchovská[3], Susana Proença[4], Trine Husøy[5], Helena Kandárová[6], Mathieu Vinken[7]
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Designed as an AI-enabled system, OPRA combines probabilistic risk assessment methods with multiple computational tools and data sources to enable fast, user-friendly, yet thorough toxicological evaluations. From a theoretical perspective, OPRA is a digital decision-tree that integrates the core components of toxicological risk assessment – hazard and exposure evaluation, biokinetic modelling to inform internal TTC-based reasoning, and biological activity insights derived from advanced, endpoint-specific in vitro systems. Due to its probabilistic nature, OPRA allows users to quantify uncertainty and variability in all individual components, producing risk estimates with confidence levels rather than single-point estimates. Where more suitable, OPRA also provides mathematical and statistical alternatives for the evaluation of the quality of evidence, as well as evidence weighting and combination for decision-making. OPRA is intended to help focus resources on “de-risking” substances with the highest probability of toxicity or requiring further laboratory investigation. This approach is particularly suitable for screening and prioritizing chemicals, when: • The number of substances awaiting evaluation exceeds current testing capacity • In vitro or in vivo testing is limited by ethical, technical, or financial constraints OPRA is currently being computationally integrated within Toxindex – a sophisticated agentic platform powered by large language models (LLMs), agents which perform a variety of tasks, and a large collection of datasets, literature, and computational tools. These features make Toxindex suitable to access, predict, process, and integrate relevant data at each step of the OPRA workflow, guide users through risk assessment tasks, and generate comprehensive reports. The probabilistic component is the next feature to be implemented, and efforts toward its development have already begun. This presentation will introduce the OPRA framework, describe the uncertainty component, and present an overview of Toxindex.

Supported by ONTOX Grant: 963845

Wednesday, 1 July 2026
18:30 - 19:30

Session ESR2: Early Stage Researcher Session 2

Chairs: *Peter Pobis (Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Slovakia) & Mohamed Fathi Abdallah (Aarhus University, Denmark)*

O-ESR2-1

Addressing the role of maternal inflammation in autism spectrum disorder using immune-competent iPSC derived brain microphysiological systems
ABSTRACT #301

Alex Rittenhouse[1], Jingyi Zhang[1], Jason Laird[1], Lena Smirnova[1]
[1]*Johns Hopkins Bloomberg School of Public Health*

Background: Autism spectrum disorder (ASD) is a major public health concern worldwide, currently affecting up to 1 in 36 children in the United States. Despite its high prevalence, the etiology of this complex neurodevelopmental disorder remains incompletely understood. Genetic factors contribute to ASD risk, including recurrent copy number variants such as 16p11.2 deletion. However, genetics alone cannot explain the rapid increase in ASD prevalence, nor the range of clinical outcomes observed among individuals carrying the same mutation, such as differences in intellectual ability and seizure occurrence. This heterogeneity suggests a key role for environmental influences. Methods: To study this, we have established an immune-competent brain microphysiological system [1] (µbMPS) that contains microglia—the resident immune cells of the brain. We differentiate µbMPS from eight 16p11.2del lines from male and female individuals with and without ASD and seizures, alongside two neurotypical control lines. This platform enables us to investigate mutation-specific changes and their interactions with inflammatory environmental exposures in a human-relevant system. Results: Maternal inflammation was modeled through exposure to pro-inflammatory cytokines in combination with fever and acetaminophen. We observed that neurotypical control microglia incorporated into 16p11.2 deletion µbMPS exhibit heightened activation following exposure. Moreover, 16p11.2 deletion µbMPS demonstrate elevated inflammatory signaling and pronounced transcriptional changes, including upregulation of immune pathways, alterations in extracellular matrix components, and downregulation of glutamatergic synapse-related genes. These effects are markedly reduced in control µbMPS. In parallel, we assessed microglia in monoculture and their capacity for synaptosome uptake. Microglia carrying the 16p11.2 deletion showed reduced baseline synaptosome uptake but responded similarly to inflammatory exposures, with cytokines exerting the strongest effect. Discussion: Collectively, these findings advance our understanding of microglial dysfunction in ASD, highlight gene–environment interactions in neurodevelopment, and establish µbMPS as a powerful tool for studying autism pathogenesis across genetic risk factors.

O-ESR2-2

Merging organoid and organ-on-chip technology to generate physiological models of ocular tissue
ABSTRACT #351

Svenja Wingerter[12], Kevin Achberger[3], Madalena Cipriano[1], Julia Roosz[4], Seyma Bektas Tercan[1], Johanna Chuchuy[1], Kaushambee Dave[3], Simon J. Clark[256], Stefan Liebau[3], Peter Loskill[14]
[1]*Department for Microphysiological Systems, Institute of Biomedical Engineering, Faculty of Medicine, Eberhard Karls University Tübingen, Tübingen, Germany*
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[4]*NMI Natural and Medical Sciences Institute at the University of Tübingen, Reutlingen, Germany*
[5]*Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK*
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Developing therapeutics for ophthalmic diseases heavily relies on animal models with limited human translatability. The emergence of human induced pluripotent stem cell (hiPSC)-derived retinal organoids (ROs) containing most major retinal cell types, provided a crucial advancement to tackle this challenge. Combined with Organ-on-Chip technology, ROs can provide even more complex human microphysiological models. We have developed a Retina-on-Chip platform that integrates hiPSC-derived retinal pigment epithelial cells (RPE) and stratified ROs to recapitulate the native human retinal architecture. The tissue is separated by an isoporous membrane from a perfusion channel, mimicking the choroidal vasculature. The proximity-culture of ROs with RPE promotes photoreceptor maturation as well as outer segment formation, shedding and

phagocytosis, reproducing in vivo recycling processes. The open-top concept allows modeling of intravenous, intravitreal and sub-retinal drug administration, making it a versatile tool for preclinical testing. The model was validated using known retinopathic drugs such as chloroquine, reproducing characteristic toxicity. The vasculature-like perfusion combined with live cell imaging allows time-resolved monitoring of tissue viability and drug responses. It has also been extensively tested for subretinal treatments using various adeno-associated viruses (AAVs), monitoring tissue viability, transduction efficiency and cell tropism. In addition, patient specific hiPSC-derived ROs have been used to model CLN2 disease pathology. AAV-based gene therapy demonstrated its potential to restore lysosomal activity and reverse disease phenotypes. Ongoing development aim to integrate bioinspired membranes, a choriocapillaris-like vasculature and immune components, creating a physiological relevant model of the outer blood-retinal-barrier and immune cell interactions in retinal diseases. Together, the RoC model enables toxicity assessment and efficacy testing of gene therapies and chemical compounds, showing its potential for preclinical evaluation. With further integration of complexity, it will facilitate modeling of a wide range of retinal diseases and will advance in vitro ophthalmic research.

O-ESR2-3

Advanced lung organoids mimicking proximal and distal regions for viral infection and drug screening
ABSTRACT #105

PhD. Alicia Reyes Valenzuela[12], PhD. Mark Turner[3], PhD. Silvia Vidal[2], PhD. Luc Mongeau[2]
[1]*PulmonaVat*
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The lung is the primary entry point and target for many lethal respiratory viruses, highlighting the urgent need for physiologically relevant in vitro models to advance drug discovery and personalized medicine1. Current lung models—including organoids—often fail to replicate the full cellular diversity and functional complexity of the human lung, particularly the interactions between proximal and distal regions2. These limitations hinder disease modeling and reduce the predictive power for drug efficacy and distribution, contributing to the high failure rate (92–96%) of drugs that succeed in animal models but fail in clinical trials3. The scarcity of comprehensive lung models has notably impeded therapeutic progress, as exemplified by the approval of only one drug for cystic fibrosis in the past 30 years. Here, we present an advanced lung organoid model generated from tracheal/bronchial epithelial cells co-cultured with feeder cells in hydrogels. Mimicking natural lung development, this system leverages epithelial-mesenchymal interactions to promote cellular and structural heterogeneity within a rapid 21-day maturation period. Multi-omic analyses (transcriptomics and proteomics) reveal upregulation of pathways involved in lung development, tube formation, and alveolar function. Structurally, the organoids exhibit tubular morphologies and microvilli, and contain both proximal (ciliated, goblet, basal) and distal (alveolar type I and II) cell types. Functionally, the organoids are infectable by both SARS-CoV-2 and influenza viruses, enabling disease modeling across lung regions. By overcoming key limitations of existing models, this platform offers a cost-effective, scalable, and physiologically relevant alternative to animal testing, poised to accelerate respiratory research and drug development.

O-ESR2-4

Organoid printing for in vitro engineering of living systems
ABSTRACT #491

Yuan Pang

Bioprinting provides a possible approach for constructing functional organ substitutes in vitro and has attracted significant attention in recent years. However, traditional printing methods based on single-cell manipulation struggle to address the challenge of reconstructing the complex microenvironment of in vivo tissues. Taking tumor tissue construction as an example, the complexity and heterogeneity of the tumor microenvironment (TME) significantly influence cancer progression and therapeutic outcomes, highlighting the need for physiologically relevant in vitro models. Addressing these problems, we proposed and

established a novel 3D printing method based on organoid assembly. Through this technology, we attempted to achieve large volume tissue with in vivo-like high cell density and functionality. This talk will show in detail of our progress in bioprinting of hepatocyte organoids to treat liver failure, and using a “cell aggregate–rich living gel” to engineer a vascularized tumor assembloids for drug screening and pathological studies.

O-ESR2-5

Silkworms as a Living NAM Bridging In Vitro Assays and Mammalian Testing

ABSTRACT #492

Atsushi Miyashita

Recent advances in new approach methodologies (NAMs), including in vitro assays, organoids, and computational models, have transformed toxicology and drug discovery. However, when efficacy, toxicity, host response, and disease context are tightly intertwined, reductionist systems alone often fail to predict in vivo performance. In this talk, I introduce the silkworm infection model as a living NAM that bridges the gap between in vitro assays and mammalian testing. Silkworms are sufficiently large to permit quantitative injection, infection, drug administration, oral dosing, and hemolymph collection, and they enable relatively high-throughput evaluation of individual-level efficacy and acute toxicity. Our previous studies have shown that silkworms can be used for oral toxicity assessment and for the identification of bioactive compounds, including the antibacterial agent lysocin E and the antifungal agent ASP2397, which was discovered through a silkworm model of Aspergillus fumigatus infection. We are now extending this platform to invasive fungal infections, particularly mucormycosis. Because in vitro antifungal activity alone cannot reliably predict therapeutic efficacy, safety, or disease-context dependence in a living host, an in vivo-first strategy using silkworms may help prioritize compounds with greater translational potential before mammalian validation. In parallel, we are developing AI-assisted and automated observation systems that quantify survival, activity, posture, and recovery trajectories from image data, thereby transforming silkworm assays into data-rich phenotyping platforms. This approach does not simply automate scoring; it creates a new methodology for continuously reading the state of an individual organism. Finally, silkworms also represent a biocultural resource rooted in the long history of sericulture across Asia. By connecting this historical foundation to contemporary NAM research, we may build a more ethical, scalable, and mechanistically informative pipeline that links in vitro testing with mammalian studies.

O-ESR2-6

Supporting Future NAMs Toxicologists: The Growing Role of the ESTIV Early Career Network

ABSTRACT #479

Mohamed F. Abdallah[1], Luiz Ladeira[2], Peter Pôbiš[3], Andreas O. Stucki[4], Bambou Xuezhu Tan[5], Benoit Maisonneuve[6], Clive Roper[7], Helena Kaňďárová[3]
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The European Society of Toxicology In Vitro – Early Career Network (ESTIV ECN) was established in 2024 to promote opportunities for early career scientists in the field of in vitro and in silico toxicology (1). The ESTIV-ECN leads four well-defined activities (a) providing career development resources and mentorship, (b) promoting networking and collaboration, (c) offering discounted conference fees and competitive awards to enhance accessibility, and (d) enabling early career members to actively participate in ESTIV decision-making processes, thereby strengthening their engagement with the society. In this poster presentation, ESTIV ECN will highlight it is key initiatives, especially the ESTIV ECN Mentorship Program. In its 2nd edition, highly motivated early career scientists from academia and industry—at different ca-reer 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 ex-pert 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 progress will be presented at the ESTIV 2026 congress to inspire young researchers attending the congress and to empower the next generation of scien-tists in the field of next-generation risk assessment. All authors are ESTIV Board Members. The authors do not necessarily represent the views of their official affiliations in this abstract. Acknowledgement The authors would like to thank ESTIV for its continuous support, as well as all volunteers involved in the organization and delivery of various ESTIV activities.

Tuesday, 30 June 2026
13:00 - 14:00

Session LS1: Connecting Early-Career Researchers: ASPIS Academy, PARC Junior Community, and VICT3R ESR network

Chairs: *Rita Ortega-Vallbona (ProtoQSAR, Spain) & Luiz Ladeira (University of Liège)*

O-LS1-1

The ASPIS academy: empowering the next generation of toxicologists ABSTRACT #16

Rita Ortega Vallbona[1], Julen Sanz Serrano[2], Anna Melina Steinbach[3], Marie Corradi[4], Eike Cöllen[5], Peter Pobis[6], Hiba Khalidi[7], Shaleen Glasgow[8], Marije Niemeijer[9], Job Berkhout[10], Eliska Kuchovska[11], Julia Dominika Zajac[2], Agata Ormanin-Lewandowska[8], Martijn J. Moné[9], Helena Kandárová[6], Silvia Tangianu[5], Giorgia Pallocca[5], François Busquet[12]
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[12]*Altertox, Belgium*

In the context of a global shift toward animal-free toxicology, early-stage researchers (ESRs) play a central role in driving innovation through New Approach Methodologies (NAMs). The ASPIS Academy (AA) is a dedicated ESR network established in 2023 within the ASPIS cluster, which unites three Horizon 2020 projects: ONTOX (1), RISK-HUNT3R (2), and PrecisionTox (3). The AA plays a pivotal role in advancing animal-free chemical risk assessment by equipping over 120 ESRs with the skills and opportunities needed to innovate in the field of toxicology. Through a diverse portfolio of initiatives, including tailored training, mentoring, lab exchange (twinning) opportunities, and science communication, the AA fosters both scientific and transferable skill development. The Academy also embraces diversity and inclusivity, ensuring that researchers from all backgrounds can actively contribute to shaping the future of toxicology. Led by ESR representatives and supported by senior scientists, the AA fosters a dynamic environment for knowledge exchange and peer support across disciplines. It empowers its members to engage with NAMs in vitro test systems, in silico tools, omics technologies, and artificial intelligence, preparing them to lead in the transition toward human-relevant, non-animal testing strategies. This presentation will introduce the ASPIS Academy as a model for ESR engagement and capacity-building and aims to open dialogue with other networks to explore synergies, shared challenges, and collaborative opportunities in shaping the next generation of toxicologists.

Supported by Horizon 2020 grant agreements No. 963845 (ONTOX), No. 965406 (PrecisionTox), No. 964537 (RISK-HUNT3R).

O-LS1-2

PARC junior community: the early-stage researcher community of the partnership for the assessment of risks from chemicals (PARC)
ABSTRACT #115

Katya Manuella Permana[1], Maria Tannous[1], Eike Cöllen[2], Rayane Boufalaas[3], Gilles Rivière[1], Eugenia Dessipri[4], Christophe Rousselle[1]
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The Partnership for the Assessment of Risks from Chemicals (PARC) is an international partnership with a total budget of €400M co-funded by Horizon Europe, set to run from 2022-2029. The consortium is made up of >200 partners from 29 countries. PARC Junior community (PJC) was founded in March 2024 under the advisory of PARC Management Board and Coordination Team. Since then, we have grown into a community of >100 members from >60 institutions across Europe. Our main goal is to establish a sense of belonging among the junior members of PARC, facilitating a space to grow professionally and personally. The PJC online lunchtime meetings provide the opportunity for members to present their work or listen to their peers discussing different topics of interest to them. We also invite guest experts to conduct webinars and share their experience of the various career paths in chemical risk assessment, giving our junior members the opportunity to connect with professionals from related fields. In addition, PJC also circulates relevant training and career opportunities through its mailing list. In this session, two PJC members who were selected through an internal short-presentation competition will give you a glimpse of the work they do under PARC related to: 1) New Approach Methodologies (NAMs) for Human Health Risk/Hazard Assessment, and 2) Physiologically Based Toxicokinetic (PBK) models and quantitative systems toxicology. We regularly hold these competitions to select representatives of our community and support them at international stages. We believe that collaborating with other junior communities is both impactful and essential for building a strong, interconnected network. PJC has joined forces with other early-stage researcher communities like SETAC SAC, ASPIS Academy and VICT3R to enrich the experience of its members. We hope that this session will motivate you to join similar initiatives or inspire you to establish one.

O-LS1-3

Introduction to VICT3R ESR network
ABSTRACT #54

Julia Matyjasiak[1], Jorge Lemos Portela[2], Jian Jiang[1]
[1]*Vrije Universiteit Brussel, Brussels, Belgium*
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The VICT3R (Developing and Implementing Virtual Control Groups to Reduce Animal Use in Toxicology Research) consortium is a public-private partnership dedicated to advancing toxicology while reducing animal use in line with the 3Rs principles. By promoting the reuse of historical control data (HCD) and developing AI-powered Virtual Control Groups (VCGs), VICT3R is pioneering animal use-reducing strategies. Early Stage Researchers (ESRs) are at the heart of this progress, bringing fresh perspectives to the field. This session highlights the growing role of VICT3R's ESRs in shaping discussions within the consortium and building a strong and supportive early career network. Inspired by the ASPIS Academy, the VICT3R ESRs also aim to create a supportive environment for growth and career development by organizing events and uniting the consortium. VICT3R ESRs have organized meaningful dialogue through seminars with experts, established open feedback channels helping to identify the evolving needs of early career scientists, and will take on moderating roles in webinars hosted by the VICT3R external communications team. A recent survey conducted by the ESRs collected ideas and priorities from across the consortium, laying the foundation for a new series of activities. These will include a mentoring scheme that connects early career scientists with experienced mentors from diverse backgrounds, including toxicology, data science, and related disciplines, a journal club that explores key

topics related to developing VCGs and other AI-powered NAMs in toxicology, workshops on career development and science communication as well as informal peer networking events. These efforts mark the beginning of a more connected and empowered ESR community that values collaboration, mutual support and scientific impact. By fostering links across the broader ASPIS cluster and other initiatives like PARC, VICT3R ESRs aim to co-create a unified early career platform that advances the 3Rs, strengthens NAMs, and shapes the future of ethical toxicology research.

POSTER PRESENTATIONS

ALL POSTER PRESENTATIONS

Bio-engineering, stem cells and disease models

P002
Assessment of hepatic fibrosis through impedance technology using a three-dimensional cell model
ABSTRACT #152

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[1]*Korea Institute of Toxicology, Republic of Korea*
[2]*University of Science and Technology, Republic of Korea*

*Background and Objectives Electrochemical impedance spectroscopy characterizes cellular properties through frequency-dependent electrical measurements. However, its application to 3D cell models faces technical challenges due to complex circuit configurations in 3D systems. This study developed a 3D hepatic fibrosis model and introduced a novel impedance-based method to quantify fibrosis-associated stiffness changes. *Material and Methods The 3D hepatic fibrosis model was constructed by co-culturing human liver cell lines HepaRG and LX-2 (7:3 ratio) on ultra-low attachment (ULA) plates for 5 days, followed by TGF-β treatment (10, 20 ng/ml) for 7 days to induce fibrosis. Fibrosis markers were analyzed by qRT-PCR and western blotting. The impedance system performed automated multi-well measurements under dynamic pressure conditions (0-100 hPa), quantifying resistance changes that correlate with stiffness. *Results TGF-β-treated 3D hepatic models showed significant upregulation of fibrosis markers including α-SMA, COL1A1, TGF-β, TIMP1, MMP2, Fibronectin, and Keratin at both mRNA and protein levels, confirming successful fibrosis induction. Impedance measurements under dynamic pressure revealed significantly reduced complex resistance values in fibrotic models relative to controls, reflecting enhanced tissue stiffness. The system's capability to detect tebuconazole-induced fibrotic changes at concentrations of 30-60 μM for 7 days further demonstrates its potential as a sensitive screening tool for identifying hepatofibrotic compounds. *Discussion and Conclusion Our findings establish impedance technology as an effective, quantitative method for measuring fibrosis-associated stiffness changes in 3D hepatic models. This real-time, non-invasive approach offers significant advantages over conventional endpoint assays, providing continuous monitoring capabilities. The technology shows promising applications for organoid-based drug screening, toxicity assessment, and fibrosis research across various biomedical fields.

P003
Characterization of stage I retinal organoids: a proteomic approach
ABSTRACT #167

Sara Tirendi[12], Anna Maria Bassi[12], Mariapaola Nitti[1], Nicole Grinovero[3], Sonia Scarfì[42], Serena Mirata[42], Mario Passalacqua[12], Fabrizio Loiacono[5], Andrea Petretto[3], Stefania Vernazza[12]
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Retinal Ganglion Cells (RGCs) are neurons that play a pivotal role in transmitting visual information to the brain via the optic nerve. The loss of RGCs is involved in several neurodegenerative eye diseases (e.g., glaucoma, anterior ischemic optic neuropathy, and retinal ischemia), causing severe visual impairment. Since human RGCs are difficult to isolate and study, advances in stem cell engineering have now enabled the use of retinal organoids (ROs) for species-specific eye studies that were previously unfeasible. ROs represent a versatile in vitro model and provide a well-organized retinal structure for exploring the mechanisms behind retinal diseases and their development. Although ROs have structural limitations, such as incomplete retinal circuits, they can provide insights into mechanisms contributing to the development or progression of retinal diseases. In our previous work, nanoscale liquid chromatography coupled with tandem mass spectrometry (nLC-

MS/MS) analysis was used for a comprehensive proteomic analysis of total cells derived from ROs, revealing a more mature protein expression pattern after the fifth week of differentiation. Furthermore, since CD90 has been reported to be a cell-surface glycoprotein expressed in retinal RGCs, we isolated the CD90+ cell population derived from ROs using the low-pressure FACS sorting. Despite its prominent expression in RGCs, CD90 is not exclusively expressed by this cell population within the retina at these time points. Indeed, proteomic data and confocal images revealed the presence of astrocytes and microglia within the CD90+ cell population, in addition to RGCs. Anyway, it cannot be excluded that these cells allow for longer culture of RGCs compared to pure cultures. More importantly, the crosstalk between glial cells and RGCs may represent a valuable resource for future retinal disease modeling. In conclusion, this study provides robust protein landscape derived from ROs, which will be helpful for future investigations.

P004
Chicken-derived R-spondin1, Wnt3a, Nrg1 and FGF1 allow branched growth of embryonal and adult chicken intestinal organoid cultures
ABSTRACT #204

Jean Paul Ten Klooster[1], Kitty Van Summeren[1], Raymond Pieters[1]
[1]*University of Applied Sciences Utrecht (UASU)*

Intestinal organoids are widely used in mammalian studies to mimic and study in vivo intestinal function and host–pathogen interactions. Recently, we have shown that embryonal intestinal organoids can grow for 10-15 passages (approximately 6 weeks) when using chicken-derived Rspo1 and Wnt3. However, adult chicken organoids did not grow under these conditions and growing these organoids for longer than 15 passages was not possible. In presented work we show that replacing Wnt3 with chicken Wnt3a and adding chicken Nrg1 and human FGF1 results in efficient and long lasting growth, over 40 weeks, of embryonal chicken intestinal organoids. Moreover, the typical branching phenotype, which is normally observed for mammalian species such as mouse and pig intestinal organoids, is now also observed in the chicken organoids when Rspo1, Wnt3a, Nrg1 and FGF1 are used. This branching is enhanced by inhibiting FoxO1 with AS1842856, resulting in an increase of growth. With this new intestinal model for chickens, feed ingredients can be tested with regard to immunological and endocrinological effects prior to field studies needed to confirm potential improvement of animal health.

P005
Bridging complexity and accessibility: A simplified organoid-derived cell model for advanced in vitro applications
ABSTRACT #207

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In vitro models are essential for studying complex biological processes and to reduce reliance on animal testing. While traditional cell culture systems based on immortalized cell lines are widely used due to their simplicity and cost-effectiveness, they lack the cellular diversity and structural complexity of native tissues. More advanced models like organoids, offer a more physiologically relevant alternative, featuring three-dimensional architecture and organ-specific cell types. However, their broader application is limited by high costs, technical complexity, and incompatibility with high-throughput workflows. In this study, we present a cost-effective method to generate organoid-derived cell lines that retain key cellular diversity while being compatible with standard 2D culture systems. Using a multi-step dissociation protocol, we transitioned 3D organoids into single-cell cultures, adapted them to 2D growth, and replaced specialized organoid media with basic culture conditions. With this method diverse organoid derived cell lines can be generated what makes this model more accessible for routine use in research and industry. We performed comparative analyses between the organoid-derived cell line and their original 3D counterparts. The results demonstrate that this

derived cell line maintains functional characteristics, including mucus and hormone secretion, barrier integrity using cell-insert assays. This model offers a practical balance between biological relevance and experimental accessibility, enabling broader adoption of complex cell systems in translational research, drug screening, and mechanistic studies.

P006
Chronic pain in a dish: modelling molecular pain mechanisms for transforming toxicity testing and drug development
ABSTRACT #229

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Chronic pain, often originating in peripheral tissues such as the skin, is a widespread problem worldwide. Its underlying mechanisms and treatment strategies are still primarily investigated in rodent models, posing both translational and ethical hurdles. This research introduces two in vitro models aimed at elucidating chronic pain pathophysiology and enhancing drug discovery and toxicity assessment. The initial model employs human induced pluripotent stem cell (hiPSC)-derived sensory neurons genetically modified to co-secrete luciferase along with substance P and calcitonin gene-related peptide - neuropeptides integral to chronic pain signalling. This setup facilitates a high-throughput cellular-level proxy for nociception. The secondary model features a three-dimensional innervated skin model (ISM), integrating fibroblasts, keratinocytes, and hiPSC-derived sensory neurons and Schwann cells. This construct is tailored to investigate chronic pain-related nerve outgrowth and gene expression changes under neuroinflammatory conditions. Initial findings confirm the successful implementation of the Luciferase assay within SiMa cells and its subsequent adaptation to sensory neurons, demonstrating consistent exocytosis induction via specific buffers. Expression of TRPV1 receptors by these sensory neurons suggests their capability to respond to TRP agonists, a crucial attribute for evaluating pain-related reactions. Within the ISM, fibroblasts subjected to ascorbic acid treatment for 21 days effectively established a stable extracellular matrix (ECM) while avoiding myofibroblast differentiation, preserving model stability. Current efforts focus on incorporating sensory neurons and Schwann cells into the ECM, alongside exploring the impact of neuroinflammation on nerve structure and genetic expression. These in vitro models represent valuable resources for probing chronic pain mechanisms, identifying potential treatments, and diminishing dependence on animal experimentation, thereby fostering more relevant and applicable outcomes for specialized toxicologists engaged in in vitro research. The project Chronic Pain in a Dish is funded by the Foundation for the Promotion of Alternatives to Animal Testing (set Foundation).

P007
Reproducible generation of robust human iPSC-derived alveolar macrophage, for in vitro lung toxicology
ABSTRACT #278

Shogo Nagata[1], Erik Johansson[1], Ariko Fuke[1], Masako Kishihata[1], Kensuke Matsumoto[1], Arun Kumar[1], Harry Choi[1], Toshio Suzuki[1], Tetsuharu Nagamoto[1], Yuki Yamamoto[1]
[1]*HiLung Inc.*

We report a human-relevant lung toxicology model, based on human induced pluripotent stem cell (hiPSC)-derived macrophage – alone, as well as in combination with hiPSC-derived alveolar epithelium – that offer both high reproducibility and robustness. Building on our respiratory and immunological engineering of hiPSCs (1), we have devised a set of protocols generating mature tissue-resident alveolar macrophage, each with expression of relevant markers such as CD169 and PPARγ, characteristic polarizations, and functional features including phagocytosis of pulmonary surfactant. Selective handling of progenitor populations allow for a reproducible model comprised of well-defined cell subpopulations. These macrophages are viable for weeks, allowing for lengthier time to conduct meaningful studies. On top of the human relevance, we demonstrate that our

liberty in being able to choose any source iPSC allows for an 'autologous' lung immunity model, in which both the macrophage and the epithelium come from the same source, thus with the same genetic background. Unlike in existing models where the macrophage population and the lung tissue (or cell line) comes from different donors, thus are not free from 'allogenic' reactions muddying the results, our model offers. With regards to co-cultured models in particular, we demonstrate that iPSC-derived alveolar macrophage exposed to ex situ toxicological injury yields fibrotic changes to the alveolar organoid. We have conducted toxicological studies, both in the context of therapeutic safety as well as industrial toxicology. The former includes therapeutics of novel modalities that entail recently-revealed risks of lung toxicity, while the latter includes novel materials and chemicals, including those exposed through inhalation. These data illustrate the human and respiratory relevance of our model, as well as the flexibility it allows in study designs. As a readily-accessible, reproducible, and scalable in vitro model, it should be of value to all researchers interested in lung toxicology that involve alveolar macrophage.

P008
Development of an in vitro assessment for drug-induced peripheral neuropathy using hiPSC-derived sensory neurons
ABSTRACT #305

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Drug-induced peripheral neuropathy is a common, dose-limiting toxicity across therapeutic areas, arising from peripheral sensory neuron injury and causing pain, numbness, and functional impairment. Conventional in vivo approaches to assessing PN are costly, require prolonged dosing and large quantities of compounds, and often fail to accurately recapitulate human phenotypes. We therefore developed and validated an in vitro assessment of drug-induced PN. We used a hiPSC-derived sensory neuron model and five readouts per cell culture plate to capture a range of PN mechanisms: imaging-based neurite length quantification, release of neurofilament light chain (NfL; a clinical biomarker), live cell staining, dead cell staining, and mitochondrial polarisation staining. The assay does not capture a demyelination mechanism of peripheral neuropathy. We validated our in vitro PN assay with clinical small molecule compounds by comparing the reported drug-induced PN incidence with in vitro toxicity observed at cmax and achieved high predictivity. The assay correctly ranked compound toxicity for TRK inhibitors (repotrectinib > entrectinib > larotrectinib) and platinum-based chemotherapies (cisplatin > oxaliplatin). While robust validation studies of conventional in vivo PN assessments in rodents and non-human primates are not available, their sensitivity is indicated to be in the range of 60-90% for high-risk compounds. Therefore, our in vitro PN assay offers comparable or superior predictivity to conventional in vivo methods, while delivering rapid results and enabling toxicity screening in an early drug discovery phase.

P009
Scalable and well-defined human CNS co-culture platforms suitable for modelling excitatory-inhibitory neuron imbalances in drug discovery and safety studies
ABSTRACT #307

Tony Oosterveen[1], Mitzy Rios de Anda[1], Tulin Tatar Ozkan[1], Sarah Hussain[1], Aurora Veteleanu[1], Stefan Milde[1], Mariangela Iovino[1], Dario Magnani[1], Tom Harris-Brown[1], Rebecca Northeast[1], Inês Ferreira[1], Amanda Turner[1], Will Bernard[1]
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Cortical neuronal circuits are composed of two principal neuronal classes: glutamatergic excitatory neurons and GABAergic inhibitory neurons (IN). Aberrant IN function and resultant excitatory-inhibitory (E/I) imbalances are implicated in various neurological disorders including autism, epilepsy, and schizophrenia. There is a demonstrable need for scalable methodologies to generate human in vitro models that reliably recapitulate E/I imbalances for high-content drug screening and toxicology assays. Leveraging our deterministic cell programming technology (opti-ox™), we have successfully generated the core cell types essential for establishing a platform to investigate E/I neuron imbalances: GABAergic

neurons (ioGABAergic Neurons), glutamatergic neurons (ioGlutamatergic Neurons), and astrocytes (ioAstrocytes), derived from human induced pluripotent stem cells. Comprehensive molecular characterisation via immunocytochemistry, RT-qPCR, and single-cell RNA-sequencing demonstrates that cultures consist of over 95% pure GABAergic neurons expressing the canonical markers GAD1, GAD2, VGAT, DLX1, and DLX2, and exhibit GABA positivity. Three independently manufactured lots of ioGABAergic Neurons showed highly equivalent transcriptomic profiles, confirming the consistency and scalability inherent to the opti-ox technology. Functional validation through Microelectrode Array (MEA) assays demonstrated that ioGABAergic Neurons inhibit the excitatory activity of ioGlutamatergic Neurons in a ratio-dependent manner, and that E/I balances can be further modulated by pharmacological agents targeting GABAergic signalling. In addition, we have developed protocols to culture ioGlutamatergic Neurons with glial cells, specifically ioOLCs (oligodendrocyte-like cells), ioMicroglia and ioAstrocytes. These models afford valuable opportunities for investigating the mechanisms underlying immune activation and demyelination, thereby facilitating drug discovery in these therapeutic areas. Furthermore, these complex multi-cell models are critical for drug safety studies as they can recapitulate key cellular interactions in the human brain. The opti-ox driven co-culture platforms offer a means to model intricate CNS interactions accurately in vitro, thereby advancing our understanding of the principles underpinning E/I imbalances and directly supporting neurotoxicity assays and drug discovery for debilitating neurological disorders.

P010
Comparative study of CYP450 gene regulation in human pluripotent stem cell-derived liver 2D cell and 3D organoid models
ABSTRACT #309

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Human pluripotent stem cell (hPSC)-derived hepatic models, including 2D hepatocyte-like cells (2D HLCs) and hepatic organoids (HOs), are valuable in vitro models for evaluating the safety and efficacy of drugs. However, 2D HLCs show limited expression and activity of drug-metabolizing enzymes, particularly cytochrome P450 (CYP450), which are involved in detoxification, a major liver function. HOs have more mature properties than 2D HLCs, particularly enhanced CYP450 gene expression. However, the transcriptional regulatory mechanisms that correlate with CYP450 expression in HOs remain unclear. Epigenetic mechanisms, including DNA methylation and histone modification, are essential for controlling gene expression during stem cell differentiation. Here, we identified epigenetic states around transcriptional regulatory regions and compared them with those in primary human hepatocytes. We found that significantly higher CYP450 gene expression in hPSC-derived HOs than in 2D HLCs was strongly associated with decreased DNA methylation and increased enrichment of histone H3 lysine 27 acetylation in their transcriptional regulatory regions. Furthermore, because of the higher expression of nuclear receptor genes, especially constitutive androstane receptor and pregnane X receptor, HOs showed higher NR-mediated induction of CYP3A4, UGT1A1, and MDR1 than 2D HLCs. Therefore, these results suggest that mature epigenetic regulation may have an impact on drug metabolism and toxicity outcomes in hPSC-derived hepatic models and, hence, be used as an indicator of model maturation. Funding: This study was supported by the National Research Council of Science & Technology (NST) [Grant No. global-25-009] and the Korea Institute of Toxicology [Grant No. 2710086924].

P011
Application of scalable hiPSC-derived liver organoids for chemical hazard screening
ABSTRACT #313

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Recent advances in organoid culture provide alternative models to animal testing for various biomedical studies. Human pluripotent stem

cell-derived hepatic organoids (hHOs) can replicate the physiological characteristics of the liver, enabling researchers to assess drug- and chemical-induced human liver injury more accurately than with animal models or hepatocellular carcinoma cells. Although current protocols allow for the long-term proliferation of various hiPSC-derived organoid models, there are a few limitations regarding their application to high-throughput screening, such as scalability and the use of the extracellular matrix (ECM). In this study, we optimized a protocol to increase the scalability of hHOs by using a bioreactor without any ECM. Morphological differences were shown in hHOs that differentiated in a bioreactor. These hHOs exhibited similar to or superior to that of hHOs that differentiated in the presence of Matrigel. The expression of hepatocyte marker genes, including drug-metabolizing enzymes and transporters, as well as bile acid synthesis, were significantly increased in the bioreactor. Consistent with this finding, we also observed significant increases in the enzymatic activities of several CYP450 isoforms and drug sensitivity. We then employed this optimized protocol to differentiate CYP1A1-mCherry hiPSCs into HOs for chemical hazard screening. The CYP1A1-mCherry hHOs were differentiated successfully in a bioreactor and were transferred to multiwell plates for chemical screening. We examined mCherry intensity to identify the agonistic activity and measured ATP content to determine cytotoxicity of the 30 chemicals. Eleven of the thirty chemicals induced cell death, and three chemicals were identified as AHR agonists. These results suggest that our organoid-based system, which employs genome editing and bioreactors, is a useful in vitro platform for high-throughput chemical hazard screening.

P012
Engineering a human hiPSC-derived 3D liver model to predict early innate immune toxicity to AAV gene therapy
ABSTRACT #346

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Recombinant adeno-associated viral vectors (rAAVs) are a platform of choice for gene therapy. However, therapies have been hindered by unpredicted on– and off-target liver immune responses, resulting in therapy failure. Early hepatic innate immunity is tightly regulated by the interactions between hepatocytes and non-parenchymal cells (NPCs). Still, the mechanisms governing this crosstalk remain insufficiently defined, hindering the development of safer vectors and robust immune toxicity assays. Herein, we developed a human 3D liver model to dissect hepatocyte and NPC contributions to the innate immune activation. To overcome primary human NPC scarcity, donor variability and immaturity of conventional human induced pluripotent stem cell (hiPSC)-derived NPCs, we hypothesized that co-culturing primary human hepatocytes (PHHs) with hiPSC-derived macrophage and endothelial precursors would promote their liver specification and better mimic the human liver innate immune microenvironment in vitro. NPC progenitors were differentiated and assessed for phenotype and function (macrophages: CD45+, phagocytosis; endothelial: CD144+, tube formation; response to Pam3CSK4, LPS, TNFα). When co-cultured in 3D with PHH spheroids in small-scale stirred-tank systems, NPCs integrated and acquired hepatic NPC phenotype, confirmed by scRNAseq, flow cytometry and immunofluorescence. Upon prototypical stimuli, the model upregulated inflammatory mediators (e.g., IL6, CXCL8, IFNB). Following rAAV8 exposure, innate responses were detected as early as 3h post-transduction. Ongoing scRNAseq analyses are delineating cell-type-specific antiviral responses and PHH-NPC crosstalk driving immune toxicity. Altogether, this model enables the mechanistic interrogation of early innate immune toxicity within a human-relevant liver microenvironment. Continued analysis will consolidate its utility as a predictive tool for assessing liver immune responses to rAAVs and other advanced therapies, ultimately supporting a safer therapeutic development. We acknowledge fundings from Boehringer

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P313
Defining a stability window for long-term, dense T47D tumour spheroids under repeated polymeric nanoparticle exposure
ABSTRACT #440

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Long-term, dense tumour spheroids are among the most informative 3D disease models, yet they are also prone to assay artefacts that can mis-rank nanoparticle (NP) effects and compromise reproducibility. A recurring issue is the reliance on single-endpoint metabolic/ATP readouts that may not reflect structural integrity or true viable cell recovery after repeated exposure. This study establishes a practical "stability window" for prolonged NP experiments by quantifying when dense spheroids remain interpretable, adapt, or fail. T47D spheroids were formed at three seeding densities (1.5–2k, 5k, 8k cells/spheroid) and subjected to repeated dosing of PLGA–PFCE NPs at low versus high exposure over extended culture. A dissociation-to-flow workflow was optimised for compact spheroids to enable longitudinal single-cell recovery and flow-based quantification of viable yield, viability fraction, and phenotype drift. These outcomes were compared against morphology and plate-based ATP/metabolic assays to assess endpoint concordance. Spheroid stability was governed by an interaction between initial density and cumulative NP exposure. Higher exposure produced earlier destabilisation in denser spheroids, reflected by reduced growth and viable recovery and consistent shifts in flow-derived phenotype, whereas lower exposure preserved assay ability for longer. Critically, ATP/metabolic signals did not reliably track flow-derived viable recovery across conditions, revealing a reproducible failure mode of single-endpoint interpretation in long-term dense spheroids. The resulting stability landscape delineates stable, adapted, and failure regimes across density and exposure. This framework provides actionable boundary conditions and a transferable workflow to improve reproducibility and interpretability of NP studies in complex 3D models. Take-home message: A stability-window map plus dissociation-to-flow quantification prevents false conclusions in long-term dense spheroid nanoparticle studies. Acknowledgements: Phoenix OITB, NWO XS, VIVID.

P314
A model to study human intestine epithelium response to probiotics
ABSTRACT #455

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The benefits of probiotics on human health have been extensively demonstrated in the last two decades. Although many clinical studies linked specific strains with gut outcomes against inflammation, the mechanisms of action are difficult to resolve in human studies. Animal models or tumor-derived cell lines are typically used to demonstrate the effects on the intestine in a more precise mechanistic manner. However, both models present documented limitations: specie-specific characteristics, and dampened innate immunity responses or lack of cell heterogeneity, respectively. To study probiotic strain-specific effects on human innate immune response, we use patient-derived intestinal organoids, which maintain the region specificity of the gastrointestinal tract, including the innate immune components, and the cell heterogeneity, including the presence of enterocytes and secretory cell lineages. By growing human organoids as monolayer and applying previously reported media compositions, we obtained an apically accessible intestinal epithelium composed of differentiated enterocytes and secretory cells. We thus built a reproducible in vitro model to

mimic the environment where the first interaction of probiotics with human intestinal cells occurs. We anticipate that the innate immune response differs along the maturation state of enterocytes and highlight adult-stem cell organoids as useful models to study in vitro probiotic strain-specific effects on human innate immune response modulation and enhancement of the intestinal epithelial barrier function.

P315
A 3D-Based Immunofluorescence Platform for Characterizing COPD Pathology and Evaluating Treatments in Whole Airway Tissue Models
ABSTRACT #462

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Chronic obstructive pulmonary disease (COPD) is a common respiratory disease, fourth leading cause of death worldwide, and characterized by an abnormal chronic inflammatory response of the distal airways, exposed to noxious particles and fumes, such as cigarette smoke and environmental pollutants. Ongoing inflammation results over time in functional alterations of airway epithelia leading to a progressive and irreversible airflow limitation. Goblet cell hyperplasia and MUC5AC mucin overproduction are hallmark features of the lung epithelium of COPD patients. Traditionally, the assessment of MUC5AC protein produced by goblet cells is conducted using an immunohistochemical (IHC) approach. This method involves sectioning tissue into thin slices, followed by selective protein staining and image-based quantification. Although powerful, IHC is tedious, time-consuming, and requires significant expertise. To overcome these challenges and avoid potential sample damage from mechanical steps, we propose a new approach that combines 3D confocal microscopic imaging of immunofluorescent-labelled whole tissue with image analysis based on an in-house algorithm. Our results indicate that in IL-13 induced COPD models, which exhibit typical overproduction of MUC5AC by metaplastic goblet cells, Lebrikizumab, an IL-13 receptor antagonist, can block the effects of IL-13. Additionally, tissue models derived from the lower airway (MucilAir™ bronchial and SmallAir™) better replicate the hyperplasia phenotype compared to nasal tissue (MucilAir™ Nasal). The described 3D-based immunofluorescence platform could complement or replace the IHC-based approach for characterizing MUC5AC-producing goblet cells.

P316
Lentiviral Optogenetic Engineering of Human 3D Neuromuscular Junctions for Disease Modeling Applications
ABSTRACT #489

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Neuromuscular junction (NMJ) dysfunction is a hallmark of several neuromuscular diseases and neuropathies. While human iPSC-derived 3D NMJ models offer a physiologically relevant alternative to animal studies, broader adoption is limited by challenges in rapidly introducing disease-relevant genetic modifications. Here, we demonstrate lentiviral delivery of a light-sensitive ion channel to generate an optogenetically controlled human 3D NMJ within a commercially available platform, expanding its utility for disease modeling. Human iPSC-derived myoblasts were co-cultured with motor neuron neurospheres on flexible posts to generate 3D NMJ tissues. Motor neurons were transduced with a blue-light–responsive channelrhodopsin (ChR2) lentiviral construct at MOIs of 1, 5, or 10 prior to tissue assembly. NMJs generated using inducible CRISPR-edited ChR2+ motor neurons served as a control. Transduction efficiency was assessed via fluorescent reporters, and functional outcomes were quantified by measuring blue-light–evoked contractile responses normalized to electrical contractions to determine functional percent innervation. Lentiviral transduction produced robust, dose-dependent reporter expression in motor neurons within 3D NMJ tissues with no evidence of toxicity. Optogenetic stimulation reliably elicited innervation-driven contractions, with the highest MOIs yielding ~110% increased blue-light–evoked twitch force and percent innervation relative to the lowest MOI.

Optogenetic responses in lentivirally transduced NMJs exceeded those in NMJs with edited motor neurons, highlighting the efficiency of this approach. This work establishes a human iPSC-derived 3D NMJ platform supporting optogenetic, innervation-driven contractility through lentiviral genetic manipulation, providing a scalable tool for studying NMJ dysfunction and accelerating therapeutic development by enabling the rapid integration of diverse motor neuron sources.

Cardiotoxicity and cardiac efficacy models

P013
Beauvericin-induced cardiotoxicity in H9C2 cardiomyocytes and zebrafish embryos
ABSTRACT #137

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Beauvericin (BEA) is an emerging mycotoxin produced by Fusarium species that frequently contaminates feed and grain-based food products, raising increasing concern regarding its potential risks to human and animal health. Given its ionophoric nature, this study hypothesized that the heart could represent a primary target of BEA-induced toxicity. In a vertebrate model, BEA exposure significantly increased heart rate and cardiac output in wild-type zebrafish embryos. To further elucidate the underlying mechanisms, rat cardiomyoblast H9c2 cells were exposed to BEA in vitro. Transcriptomic profiling revealed enrichment of pathways related to DNA replication, cell-cycle regulation, and DNA-damage responses. RT-qPCR analysis confirmed a marked downregulation of DNA replication–associated genes, including Cdc6 and Mcm2–7. Consistently, reduced PCNA expression and decreased EdU/BrdU incorporation demonstrated strong inhibition of DNA-replication activity. Micronucleus formation assays further indicated elevated genotoxic stress following BEA exposure. At the molecular level, short-term exposure to BEA markedly increased pCHK1 (Ser345) and γH2AX (Ser139) expression, suggesting rapid induction of replication stress and activation of DNA-damage–response signaling. Collectively, these findings demonstrate that BEA exposure induces cardiotoxicity in zebrafish embryos and triggers replication-stress–associated DNA damage in rat cardiomyoblasts, providing molecular insights into the mechanisms underlying BEA-induced toxicity.

P014
Could succination lead to cardiotoxcity
ABSTRACT #380

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Cardiovascular diseases (CVDs) are a major global health challenge, leading to high rates of illness and death. Conditions like heart failure, atherosclerosis, and ischemic cardiomyopathy not only shorten lives but also seriously affect the well-being of those who live with them. To improve treatment options, we need to better understand the molecular processes that go wrong with these diseases. SUMOylation is a type of post-translational modification where SUMO proteins attach to other proteins to regulate their activity. This mechanism is especially important in the heart. For example, SUMO1, a member of the SUMO protein family, helps regulate SERCA2a, a pump that controls calcium levels and ensures the heart muscle relaxes properly after each contraction. When SUMO1 function is disrupted, SERCA2a's function is also affected, which can contribute to cardiotoxicity. In our study, we looked at how another process called succination, which occurs when the Krebs cycle metabolite, fumarate, is highly concentrated in cells and reacts with proteins. Which might interfere with SUMO1's ability to modify SERCA2a. By enriching AC16 cardiomyocytes with sodium fumarate, we observed changes in metabolism using ^{1}H NMR spectroscopy. Our findings through Western blotting suggest that succinated SUMO1 was less effective at modifying SERCA2a. This suggests that fumarate accumulation could impair calcium metabolism in cardiomyocytes, potentially leading to toxic effects. Our findings reveal a new link between two protein modifications, succination and SUMOylation, and how they may work together to affect heart health. This insight opens the door to new ways of thinking

about and possibly treating cardiovascular diseases in the future.

P015
Action potential waveform analysis in human iPSC-cardiomyocytes enables mechanistic assessment of multichannel cardiac effects
ABSTRACT #387

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Regulatory developments, including the FDA Modernisation Act 2.0, have accelerated the shift towards human relevant New Approach Methodologies (NAMs) for non clinical safety assessment. Within cardiac safety testing, traditional reliance on single ion channel assays (most notably hERG) can overlook the integrated electrophysiological interactions that contribute to proarrhythmic liability. Human induced pluripotent stem cell derived cardiomyocytes (hiPSC CMs) offer a physiologically meaningful platform capable of capturing multichannel cardiac effects in a human cellular context. This study examined whether optical voltage imaging of hiPSC CM action potentials (AP) can identify electrophysiological effects beyond hERG inhibition alone and support mechanistic interpretation when integrated with ion channel electrophysiology data. hiPSC CMs (Fujifilm CDI) were plated onto coated 96w plates and cultured for eight days before recordings. Aps were measured using the Volta fast kinetic plate reader and the voltage sensitive dye BeRST. Following dye loading and equilibration in recording buffer, fluorescence signals (10kHz) were acquired at 28°C. Reference compounds (30mM stocks) were tested across ten concentrations (upto 30µM), with repeat measurements at 24h. AP duration parameters, beat rate, and rise time were quantified using Volta analysis software. Waveform alterations were compared with established in house human ion channel datasets generated via automated patch clamp. Optical voltage imaging revealed clear, concentration dependent and compound specific modifications to AP morphology, aligning with known ion channel pharmacology. The compound panel encompassed selective potassium and calcium channel blockers as well as agents with broader multichannel activity. Triangulation demonstrated robust sensitivity to compound mechanism, increasing with exposure for multichannel modulators in line with reduced 88ioreactor88ion reserve. Lower risk hERG blockers, such as moxifloxacin, produced minimal triangulation relative to dofetilide. Across the concentration range, triangulation distinguished compounds with similar AP prolongation but differing mechanistic profiles, and waveform signatures were consistent with in house ion channel electrophysiology data.

P016
Development and characterization of a high-throughput in vitro human heart model: implications for future pre-clinical cardiac safety screening
ABSTRACT #432

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Pre workout supplements are a popular category of food supplements, and their use has risen over the past years. They are commercialized without mandatory preclinical cardiac safety testing, despite often containing pharmacologically active compounds (PACs) structurally related to (nor)adrenaline. The safety assessment of PACs has traditionally relied on animal studies, but there is an increasing demand for human-relevant non-animal methods. With a continuously growing and dynamically changing market, there is an urgent need for robust, human-based in vitro platforms that enable systematic cardiac safety evaluation of these and other emerging compounds. Current cardiac in vitro models

primarily rely on time-consuming qualitative read-outs, such as video-based contraction detection. Therefore, the aim of the current study is to develop a human-relevant high-throughput in vitro heart model with a quantitative read-out. A three-dimensional human heart model was generated by combining human embryonic stem cell-derived cardiomyocytes and fibroblasts. A customized force-measurement set-up allowed continuous assessment of contractility in real-time, providing an integrated functional read-out. Applicability of the model for pharmacological safety testing was evaluated using adrenaline. The cardiac rings show spontaneous contractions at 40-80 beats per minute (bpm), comparable to resting human heart rate, confirming the physiological relevance of the platform. Adrenaline increases beating frequency from 44 bpm to 77 bpm in the cardiac rings, mirroring the in vivo response to sympathetic stimulation and supporting functional validation of the model. Future work will employ this platform to investigate the cardiac safety of commonly used pre workout supplement ingredients and commercial products, focusing on PACs and mixtures with reported or suspected β_1 adrenergic activity. We will assess effects on contraction force, frequency and rhythm regularity, both at baseline and during adrenaline-induced "exercise" conditions, to explore its utility as a non-animal, high-throughput approach for cardiac safety assessment of pre-workout supplements.

P317
HUMAN CARDIAC ORGANOID–MEA PLATFORM FOR HUMAN-RELEVANT CARDIOTOXICITY SCREENING
ABSTRACT #468

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Cardiotoxicity remains a leading cause of drug attrition, underscoring the urgent need for predictive, human-relevant preclinical evaluation systems. Conventional animal models frequently fail to recapitulate human myocardial electrophysiology, contributing to persistent gaps between preclinical and clinical outcomes. New approach methodologies (NAMs) have emerged as physiologically relevant alternatives, a direction actively supported by regulatory frameworks such as the FDA's ISTAND Pilot Program. Among these approaches, human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes offer considerable promise; however, conventional 2D monolayer systems remain constrained in their ability to replicate the structural and electrophysiological complexity of native human myocardium. To address these limitations, we developed an integrated platform that combines 3D human cardiac organoids with a microelectrode array (MEA) system. Platform performance was validated using Comprehensive in vitro Proarrhythmia Assay (CiPA) reference compounds spanning well-defined proarrhythmic risk categories. Corrected field potential duration (FPDc) served as the primary electrophysiological endpoint, supplemented by spike amplitude and beat period to construct a comprehensive functional profile. Drug-induced electrophysiological patterns were consistent with CiPA-defined risk classifications, reliably recapitulating risk-stratified repolarization trends across high-, intermediate-, and low-risk compound categories. This organoid-MEA platform demonstrates practical scalability and reproducibility while offering enhanced physiological fidelity over existing in vitro systems. Aligned with current NAMs-based regulatory directions, it provides a robust foundation for early-stage cardiotoxicity screening and broader drug safety applications, representing a meaningful step toward next-generation cardiac safety assessment.

Case studies for successful use and implementation of complex in vitro models

P017
Advanced integrated human in vitro approach to explore gut-brain barrier interactions with gut microbiota metabolites and pesticides
ABSTRACT #141

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Chronic exposure to food pesticide residues represents a growing toxicological concern, as these compounds can affect multiple physiological systems beyond their intended targets. Among the most sensitive, the intestinal and blood–brain barriers (IB and BBB) play critical roles in maintaining host protection and homeostasis. Disruption of these barriers has been associated with increased permeability, neuroinflammation, and systemic toxicity. Recent evidence suggests that such effects may be mediated, through alterations in the gut microbiota (GM) and its metabolites, which act as key modulators of host barrier integrity. Although in vitro models are increasingly employed to assess pesticide toxicity, reproducing the complex interactions between the GM and human barriers remains challenging. This study presents an integrated in vitro strategy combining three complementary human-relevant models to investigate the microbiota-mediated impact of pesticide exposure on intestinal and brain barriers. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) was used to reproduce GM activity under both control and pesticide-exposed conditions. SHIME® supernatants containing microbial metabolites and residual pesticides were applied to a Caco-2-based IB model and a BBB co-culture model in a dual experimental setup (SHIME–IB and SHIME–BBB), allowing assessment of barrier-specific effects. The GM was treated for 15 and 30 days, enabling evaluation of both short- and long-term responses. Our results indicate that pesticide-exposed GM metabolites induce discontinuities in zonula occludens-1, 90ioreacto and claudin-5, at tight junctions and trigger inflammatory responses, without overt cytotoxicity after 24 hours of cell exposure. Transcript analysis showed a mRNA P-gp decrease, confirmed at the protein level. Overall, this integrated approach provides a relevant and ethical platform to study the microbiota-mediated effects of environmental contaminants on human barriers. It highlights the importance of the GM in modulating pesticide toxicity and offers a promising strategy for evaluating chronic exposure scenarios in a human-relevant, in vitro context.

P018
Use of advanced NAMs for food-related nanotoxicology: a case study to investigate synthetic amorphous silica (SAS) toxicity in a liver organoid model ABSTRACT #160

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New Approach Methodologies (NAMs), supporting more human-relevant and mechanistic toxicity testing, are moving nanomaterial risk assessment forward. The NAMs4NANO project funded by the European Food Safety Authority (EFSA) aims to explore the implementation of some selected NAMs in integrated approaches to testing and assessment (IATAs) to strengthen risk assessment and to address the remaining challenges and uncertainties. In this project, we developed and applied an advanced in vitro liver model combining differentiated HepaRG hepatocytes with primary human macrophages M1 to figure out the role of immune cells in any potential nanomaterial hepatotoxicity. We studied synthetic amorphous silica (SAS) that includes a group of materials authorized as food additive (E 551) and used in food industry as anticaking agent, thickener and carrier of flavors. An initial assessment was first conducted on each cell type to investigate internalization and toxic effects (cell viability, oxidative stress and genotoxicity) after a 24

hours treatment with two SAS, NM-200 and NM-203. The two types of particles were internalized in macrophages M1 as well as induced some toxicity. In contrast, 3D HepaRG cells were not affected by SAS and showed a lower level of internalization. However, the 2 SAS were cytotoxic in a model combining macrophages M1 and 3D HepaRG cells. Selective depletion of macrophages abolished the toxicity, indicating the central role of immune cells in the toxic response. These findings emphasize the need to integrate immune cells, and therefore more complex in vitro models, to improve the prediction of in vitro toxicity testing. Such advanced co-culture systems show promise for nanosafety assessment. Disclaimer : This research is performed under the NAMs4NANO action, which received funding from the EU through a grant of the EFSA (GP/EFSA/MESE/2022/01). This communication reflects only the author's view and EFSA is not responsible for any use of the information it contains.

P019
A 3D co-culture model simulating the human hepatic microenvironment
ABSTRACT #171

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A hepatic co-culture model was developed to simulate the hepatic microenvironment, in particular the liver sinusoid and space of Disse. Therefore, a Transwell-based system was selected to imitate the microarchitecture of the hepatic sinusoid and the adjacent hepatocytes. The model combines endothelial and immune cells (HMEC-1 and THP-1) on the apical compartment, and hepatocyte-like and hepatic stellate cells (HepaRG and HHSteC) on the basolateral compartment, forming the functional space of Disse in between. This indirect 3D co-culture system facilitates paracrine signaling and intercellular communication even in the absence of direct cell–cell contact. During the optimization phase, suitable cell lines, different cell ratios, seeding densities, and co-culture media were evaluated to identify optimal co-culture conditions. The final model comprised of two million cells, ensuring optimal confluence without overgrowth. The model was maintained for 48 hours with cell type ratios resembling ratios found in in vivo liver samples. Functional validation of the model was carried out by morphological assessment, fluorescence imaging of cell-specific markers, quantification of liver-specific functions (urea production, albumin secretion), as well as mRNA expression analysis of cell-specific genes. To further demonstrate the applicability and advantages of the model, the co-culture was treated with three reference compounds known to target distinct liver-related endpoints: inflammatory response, fibrogenesis (extracellular matrix formation), oxidative stress and xenobiotic metabolism. The system exhibited endpoint-specific molecular and functional responses, confirming its suitability for studying complex multicellular interactions and hepatic pathophysiological mechanisms in vitro. In summary, this hepatic co-culture provides a promising human surrogate model for investigating liver physiology, disease mechanisms, and compound-induced effects.

P020
Understanding regulatory uptake of NAMs: a multicriteria review of 20 case methods
ABSTRACT #180

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The European regulatory framework has evolved to support the use of New Approach Methodologies (NAMs) for safety assessment of chemicals and pharmaceuticals. However, their regulatory acceptance and practical use remain limited. Previous efforts and initiatives using diverse approaches e.g., qualitative interviews, online surveys, workshops, and panel discussions involving all stakeholders, have provided insights into the drivers and barriers affecting the regulatory uptake of NAMs in the broad context. Expanding these

previous efforts, the EU funded NAMWISE project aims to advance this understanding through a comprehensive assessment of how individual NAMs progressed, ultimately leading to (or were progressed to obtain) regulatory implementation. To achieve this, we gathered insights from experts across academia, industry, and regulatory bodies, leading to an initial compilation of 165 NAMs developed to replace vertebrate testing. This list was subsequently refined to ensure a balanced representation across various toxicological endpoints and methodological approaches, resulting in the selection of 20 NAMs for detailed evaluation. A comprehensive multi-criteria assessment approach was developed to assess potential drivers and barriers influencing NAM maturation across key stages of their lifecycle i.e., research, proof-of-concept, availability, validation, and regulatory use. The analysis combined literature review with expert consultation to ensure robust and contextually grounded results. The findings aim to inform regulatory authorities and stakeholders about critical factors that facilitate or hinder the regulatory implementation and use of NAMs, offering guidance relevant to both currently available NAMs and future developments in the field. This project has received funding from the European Health and Digital Executive Agency under grant n°101191595

P021
B-carotene nanocarriers as a tool to sustain hyaluronic acid and counteract skin ageing: A case study
ABSTRACT #247

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Background and Objectives Skin ageing, caused by intrinsic and extrinsic factors, results in reduced elasticity, dehydration, and depletion of hyaluronic acid (HA), a key macromolecule responsible for maintaining skin hydration and turgor [1]. Current anti-ageing treatments often rely on topical HA or dermal fillers that offer only transient effects without restoring endogenous synthesis [2]. This study aimed to develop a nanotechnology-based strategy using β-carotene-loaded solid lipid nanoparticles (SLNs) to stimulate natural HA production in skin cells and counteract ageing processes. Materials and Methods SLNs were produced by high-shear homogenization followed by sonication, employing cacao butter or Imwitor® K900 as lipid matrices and β-carotene as an antioxidant and photoprotective agent. Nanoparticles were characterized for size, polydispersity index (PDI), zeta potential, encapsulation efficiency (EE), and storage stability. Cytocompatibility, cellular uptake, and HA quantification were evaluated in HaCaT keratinocytes and human dermal fibroblasts (HDFs) using the AlphaLISA® assay. Results Optimized formulations exhibited particle sizes between 150–250 nm, PDI < 0.3, and zeta potentials from −17 to −24 mV, with EE ranging from 49% to 60%. The SLNs remained physically stable for at least 28 days at 4 °C. Both β-carotene-loaded and empty nanoparticles demonstrated high biocompatibility and were efficiently internalized by HaCaT and HDF cells. Treatment with β-carotene-loaded SLNs significantly increased intracellular and extracellular HA levels compared to untreated controls, confirming their bioactivity in enhancing HA synthesis. Discussion and Conclusion The developed β-carotene-loaded SLNs provide a safe, non-invasive, and sustainable platform to promote endogenous HA production. By combining the antioxidant potential of β-carotene with lipid-based nanocarriers, this approach bridges nanoscience and skin biology, offering a promising alternative to conventional HA supplementation for skin rejuvenation and hydration restoration.

P022
Blocking complement as a preventative measure for kidney ischemia reperfusion injury: insights from a human kidney-on-a-chip study
ABSTRACT #262

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Delayed graft function (DGF), a prevalent complication following kidney transplantation, is often driven by ischemia reperfusion injury (IRI), with complement activation playing a key role. Despite this recognition and its significant impact on transplant outcomes, preventative treatments are currently not available. In this study, we investigated the therapeutic potential of ARGX-117, a novel humanized anti-C2 antibody, in diminishing complement-induced IRI using a human kidney on-a-chip model. We established a 3D kidney-on-a-chip co-culture model in the OrganoPlate platform that incorporates human renal proximal tubule epithelial cells (RPTECs) and human umbilical vein endothelial cells (HUVECs) cultured under flow and in direct contact with a collagen-I ECM gel layer. Ischemia-reperfusion (IR) conditions were established by culturing the cells under low oxygen and without perfusion flow for 48 hours, followed by restoring normoxic conditions for 24 hours representing reperfusion. Under IR conditions, the model exhibited hallmark features of IRI, including altered RPTEC morphology, decreased barrier function, and IgM-mediated complement activation, as evidenced by C3 deposition. Furthermore, complement-driven upregulation of ICAM-1 expression on RPTECs was observed, indicative of an inflammatory response. Therapeutic intervention with ARGX-117 effectively prevented IR-induced complement activation in a concentration-dependent manner, as demonstrated by reduced C3 deposition, while preserving IgM binding. Finally, ARGX-117 attenuated the IR-induced upregulation of ICAM-1 on RPTECs, suggesting reduced inflammation and subsequent tissue damage. These findings highlight the crucial role of complement activation in IRI pathogenesis and demonstrate the efficacy of ARGX-117 using a preclinical human kidney IRI model. By preventing complement-driven inflammation and kidney damage, ARGX-117 offers a promising strategy to prevent DGF and to improve kidney transplantation outcomes. This study emphasizes the importance of advanced human in vitro models for preclinical evaluation of targeted therapies, paving the way for better management of transplantation-associated IRI.

P023
Adapting RNA-seq as an alternative acquisition platform for gene expression quantification in the GARDskin assay
ABSTRACT #272

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The GARDskin assay, included in the OECD test guideline 442E, is a non-animal method for identifying skin sensitizers. It predicts sensitization potential from the expression pattern of a 196-gene biomarker signature, traditionally quantified using NanoString nCounter technology. While nCounter offers high reproducibility and straightforward data handling, exploring alternative gene expression platforms could enhance accessibility and enable broader analyses. This study investigated whether RNA sequencing (RNA-seq) can be used as an alternative platform for gene expression quantification within the GARDskin assay. The study comprised two parts. In the first part, 24 paired samples analyzed with both nCounter and RNA-seq were used to develop an RNA-seq quantification pipeline optimized to reproduce nCounter-like gene expression profiles. This involved a primary mapping between RNA-seq transcripts and nCounter probe targets based on sequence alignment and a subsequent optimization to reconstruct the nCounter signal. Concordance between platforms was evaluated using correlation metrics and linear regression. In the second part, the predictive capacity of RNA-seq-derived data was evaluated. 24 new treatments (14 sensitizers, 10 non-sensitizers) were analyzed using RNA-seq. Processed expression data were analyzed with the standard GARDskin classification pipeline, and predictions were compared with reference labels and historical nCounter results. The RNA-seq data showed high concordance with nCounter results. Normalized expression values yielded Pearson and Spearman correlations of 0.69 and 0.66, respectively. Effect estimates between positive and vehicle controls also showed a strong linear relationship along the identity line (Pearson 0.87, Spearman 0.95, Lin's concordance 0.87). RNA-seq–derived classifications achieved 100% accuracy, sensitivity, and specificity, fully matching historical GARDskin outcomes. In conclusion, RNA-seq can be effectively adapted for gene expression quantification in the GARDskin assay. The high concordance and predictive

accuracy support RNA-seq as a viable alternative platform, broadening methodological flexibility and analytical opportunities for GARDskin.

P025
Novel 3D-printed cell culture inserts for advanced in vitro skin regeneration
ABSTRACT #329

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In skin research, 2D monolayer in vitro models provide only limited fields of application as they do not sufficiently mimic physiological properties. Since in the spirit of 3R's (Replacement, Reduction, and Refinement) animal models are to be avoided, new approaches like 3D skin equivalents (SE) are needed to close the in vitro/in vivo gap. Cell culture inserts to produce SE are commercially available, however, these inserts are expensive and limited regarding the experimental setup. The aim of this study was to design and produce novel cell culture inserts fabricated on commercially available 3D-printers to generate full thickness human skin equivalents. A computer-aided design model was realized with extrusion-based 3D printing of polylactic acid filament. In consecutive steps the design of the inserts was improved and the feasibility confirmed in cell culture experiments. Cytotoxic effects of the final product were excluded by testing the inserts according to ISO-norm procedures. Finally, to verify usability, full thickness human skin equivalents were created on collagen/fibrin dermal scaffolds which were placed in the inserts. Histologic analysis proved the comparable quality of the constructs compared to commercially available products. In conclusion, here we demonstrate an efficient and cost-effective alternative to produce 3D-printed inserts for the generation of skin equivalents. The system is feasible with common 3D printers and allows high flexibility in the choice of printing materials, membrane materials as well as of shape and size. It is compatible with commonly used cell culture plates and easy to handle. Further studies will be performed with skin equivalents based on the presented technology.

P026
Continuous in vitro cultivation of a defined human gut microbiota: assessing media-driven shifts using the peristaltic mixed tubular bioreactor (PETR)
ABSTRACT #333

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Investigating the complex human gut microbiota in vivo presents significant technical and ethical challenges. To address these limitations, there has been an increased focus on in vitro models utilizing defined microbial consortia. The Peristaltic mixed Tubular 94ioreactor (PETR) [1] is a novel in vitro system designed for the continuous cultivation of intestinal microbiota under conditions that closely mimic the human colon. Unlike traditional in vitro models, PETR incorporates a physiologically relevant tubular geometry, peristaltic mixing, a pH gradient, and the resorption of water and metabolites, in addition to controlled anoxic conditions. Using the Oligo-Mouse-Microbiota (OMM12) consortium, PETR achieved microbial stability within six days and demonstrated physiological similarities to in vivo data from gnotobiotic mice colonized with the same OMM12 community [2]. Utilizing PETR, we investigate how variation in the cultivation media impacts the gut microbiota dynamics. To simulate different levels of nutritional complexity within the human gut ecology, we selected three distinct media: Brain-Heart Infusion (BHI), Complex Intestinal Medium (CIM), and Gut Microbiota Medium (GMM). A modified, 9 strain extended version of the simplified human microbiota (SIHUMIx) [3] is used as an in vitro model for the human microbiota. Initially, we determine the specific growth rates of the SIHUMIx strains across all three media, followed by mapping their pairwise interactions through spent media cultivation. Subsequently, the SIHUMIx consortium is cultivated in PETR in continuous operation in each medium. By analyzing short-chain fatty acid (SCFA) profiles and tracking microbial composition via qPCR, we evaluate the community's response under human-like physiological conditions. Ultimately, this work validates PETR as a robust, physiologically

relevant platform for pre-clinical gut microbiota research, presenting a highly viable alternative to traditional animal testing.

P027
Development and application of an immunocompetent in vitro stem cell-based intestinal model for hazard assessment; testing the effects of faba bean, cricket and yellow chlorella proteins
ABSTRACT #359

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The intestine has an important barrier function and represents the largest compartment of the immune system, making it an important organ to consider for food safety studies. The human induced pluripotent stem cell (hiPSC)-derived intestinal epithelial cell (IEC) model is an advanced in vitro model that reflects the human intestinal epithelium more closely than cell line-based models, but it still lacks immune cells. The aim of this study was to incorporate human immature dendritic cells (iDCs) into the IEC model and to apply the model in a case study investigating (immune) effects of three novel protein sources. To establish a co-culture, various culture parameters (e.g. different medium) were evaluated and barrier integrity and measurement of differentiation and inflammation markers were used as readouts. The established co-culture model exists of IECs that were differentiated on the basolateral side of a transwell membrane. After differentiation, iDCs were added to the apical chamber, and after 24h of acclimatization, the co-culture model was exposed basolaterally to Faba bean, Cricket, and Yellow Chlorella proteins. For this, isolation and exposure protocols for proteins from food sources were established and suitable benchmark proteins were selected. After 24h exposure, no effects of the proteins on barrier integrity were observed in the co-culture model (TEER, LY transport). Additionally, no to minimal effects on cell viability (>80% viability; WST-1) were detected, depending on the concentration (5, 10, 50 µg/ml). Immune-related effects are currently being analysed. In summary, an immunocompetent hiPSC-derived IEC/iDCs intestinal model was developed and applicability of the model was shown in a case study evaluating the effects of three novel protein sources. The co-culture model was shown to be robust and reproducible and is a promising novel in vitro model to study effects of food-related compounds on the intestinal barrier.

P028
A targeted in vitro approach to fill hazard data gaps in the context of safe and sustainable by design (SSBD): a flame retardants case study
ABSTRACT #360

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The European Safe and Sustainable by Design (SSbD) framework helps industry to produce safer and more sustainable chemicals and materials, taking into consideration safety and sustainability along the entire lifecycle of the product. The framework is applied alongside the innovation process, and the maturity of the assessment evolves as the readiness of the technology grows. The framework's safety assessment steps are largely based on the use of already existing hazard data. For novel chemicals and materials, data gaps are therefore inevitable. Currently, little guidance is available on how to fill these data gaps using an approach that is compatible with the innovation process, and its constraints regarding time and resources. Here we present an approach to screen hazards of flame retardants (FR) in a feasible and cost-effective manner using new

approach methodologies (NAMs). Some FR, like hexabromocyclododecane (HBCD) and decabromodiphenyl ether (DecaBDE), have been shown to be persistent, bio accumulative, and are potentially toxic to humans and the environment, having suspected carcinogenic, mutagenic, and reprotoxic (CMR) and endocrine disrupting (ED) properties. The in vitro approach presented aims to assess CMR and ED properties of FR. For this purpose, the micronucleus assay, 53BP1 assay, mouse lymphoma assay, and cell transformation assay are combined with endocrine disruption reporter assays, to allow for the screening for genotoxic events, the progression of mutations into cell transformation and carcinogenicity, non-genotoxic pathways towards carcinogenicity, and endocrine disruption. We present the outcomes of these assays with commercially available legacy FR with known in vivo toxicity, consisting of halogenated and non-halogenated FR. The next step of this research will be to apply the testing strategy to SSbD alternative FR produced in the European project PLANETS (GA 101177608). These FR will be designed with the aim of reducing toxic effects whilst still keeping sufficient flame retarding properties.

P029
Epivaginal™ in Europe: cross-site reproducibility and discriminatory performance using ET-50 and multi-endpoint readouts
ABSTRACT #416

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Background and Objectives: EpiVaginal™ (VEC-100; MatTek) is a highly differentiated 3D human vaginal epithelium derived from normal ectocervical epithelial cells and proposed as an in vitro alternative to the Rabbit Vaginal Irritation (RVI) test. While produced robustly in the United States for many years, it was only recently introduced in Europe. This case study asked: (i) can European manufacturing meet the same batch reproducibility standards as the long-established U.S. site, and (ii) once established, does the method transfer across laboratories while retaining the ability to distinguish irritant from non-irritant response profiles? Methods: Manufacturing robustness was assessed using routine QC parameters, including ET-50 to 1% Triton X-100 (acceptance 0.70–1.70 h), average exposure CV (%), and histology, across 2024 U.S. lots (n=20) and 2024–2025 Europe lots (n=40). Method transferability was evaluated by running matched irritation testing (ET-50 and TEER) in-house and in a European partner laboratory using a shared panel of vaginal ingredients. To increase physiological relevance, selected ingredients were profiled internally with and without Simulated Vaginal Fluid (SVF) using ET-50, TEER, and inflammatory cytokines. Results: All 60 lots met ET-50 acceptance criteria with intra-lot dose–response variability <10%, supporting successful transfer of manufacturing robustness from the U.S. to Europe. Inter-laboratory testing showed strong concordance (mean differences 7.2% for viability and 6.4% for %TEER) and consistent classification of irritants versus non-irritants across sites. In SVF-conditioned studies, response patterns suggested attenuation of acidity-driven injury for selected ingredients, whereas surfactant-type injury remained largely unchanged; cytokine profiles were consistent with previously published patterns. Conclusions: Overall, EpiVaginal™ was successfully implemented in Europe with U.S.-equivalent QC reproducibility and demonstrated inter-laboratory reproducibility of the ET-50 assay and TEER readouts, enabling decision-relevant discrimination of irritant potential. Multi-endpoint testing under SVF-conditioned exposures provides a microenvironment-aware layer, and cytokine analysis adds complementary mechanistic context—together supporting animal-sparing local toxicity screening.

P030
Enhanced oxygen supply enables sustained function of primary human hepatocyte spheroids

ABSTRACT #459

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Background Three-dimensional hepatocyte spheroid models are widely used in in vitro toxicology due to their physiological relevance. However, high oxygen consumption often leads to insufficient oxygen diffusion, resulting in hypoxia and functional deterioration within the spheroid core, limiting their applicability. Methods We developed a silicone-based culture substrate with a surface-area-enhancing structure to improve oxygen diffusion from the culture medium to hepatocyte spheroids. Primary rat and human hepatocytes were cultured as spheroids on this substrate. Hepatic function was evaluated by albumin secretion. Hypoxia was assessed by HIF-1α expression, and oxygen concentration in the culture medium was measured and compared with conventional substrates. Results The oxygen-permeable, structured substrate mitigated hypoxia within hepatocyte spheroids, as indicated by reduced HIF-1α expression compared with conventional culture systems. Oxygen levels in the culture medium remained stable. Rat hepatocyte spheroids maintained morphology and sustained albumin production over extended culture. Importantly, primary human hepatocytes cultured on this platform showed high albumin secretion maintained up to at least day 7, demonstrating improved functional stability. These effects were not observed on flat silicone substrates, indicating that structural design, rather than material alone, is critical for enhanced oxygen delivery. Conclusion This study demonstrates that a surface-area-enhancing silicone substrate improves oxygen supply and supports long-term functional maintenance of hepatocyte spheroids. The platform shows potential as a human-relevant in vitro system for hepatotoxicity assessment and NAM-based applications.

Challenges in cosmetics safety

P031
Safety assessment of botanicals and natural complex substances
ABSTRACT #230

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Background and Objectives Next-generation risk assessment (NGRA) is a modern approach to chemical safety that combines in vitro assays with computational methods to understand toxicity. It identifies the concentration at which biological effects begin (PoDs), uses models to estimate internal exposure (Cmax), and calculates bioactivity-to-exposure ratios (BERs) to assess safety margins. Current approaches are primarily developed for single substances, with limited validation for complex mixtures. This gap poses challenges for risk assessment, as mixture effects remain largely unexplored. Botanical ingredients have a long-standing history in cosmetic applications and are currently experiencing renewed interest. From a toxicological perspective, evaluating new botanicals presents unique challenges due to their complex and variable compositions, which are frequently not fully characterized. The potential for mixture effects—often overlooked—adds further complexity. Materials and Methods As an initial step a detailed phytochemical analysis of the botanical of interest was developed, focusing on the identification of major constituents within the mixture. The NAM toolbox includes well-established assays for local toxicity endpoints (e.g., skin and eye irritation, skin sensitization) and systemic and reproductive toxicity assessments (like high throughput transcriptomics, ToxTracker and DevTox quickPredict) using both cell-based and cell-free models. These assays span various tissues and modes of action and are complemented by physiologically based pharmacokinetic (PBPK) modeling to estimate internal exposure to key analytes. Results Initial results of the NAM toolbox for botanical mixtures will be presented. The analysis of key components within the mixture has supported the development of a targeted testing strategy for the botanicals of interest, addressing relevant biological and toxicological endpoints. These results will further contribute to the creation of an adapted NGRA framework specifically tailored to the assessment of botanical mixtures. Discussion and Conclusion Botanicals require a tailored combinatorial approach for risk assessment, integrating

NAMs with historical usage data and detailed chemical analytics.

P032
Reciprocal interaction between PPARγ and CEBPα in lipid metabolism regulation: hypothesis of a self-sustaining regulatory loop in HaCaT cells
ABSTRACT #276

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Background Lipid metabolism is regulated by interconnected transcriptional networks that coordinate cellular differentiation and energy homeostasis. Among the main transcription factors involved, PPARγ and CEBPα are known to act cooperatively in the control of adipogenic differentiation and lipid-related gene expression. Evidence from the literature indicates a mutual activation loop, where increased expression of one factor leads to the upregulation of the other. However, the mechanisms sustaining their expression once the initial stimulus is removed remain poorly understood. Aim This study aims to investigate the reciprocal regulatory relationship between PPARγ and CEBPα in HaCaT cells, focusing on the dynamics of gene regulation and potential mechanisms that maintain their expression following transient stimulation. Methods HaCaT cells are exposed to FOS treatment to induce lipid metabolism–related pathways. The expression levels of PPARγ and CEBPα will be analyzed through gene and protein expression assays at different time points during and after stimulation. The study will assess whether a feedback mechanism may be responsible for the sustained regulation of these transcription factors once the stimulus is removed. Discussion This work aims to propose a model of autoregulatory feedback between PPARγ and CEBPα, potentially explaining how lipid metabolism–related transcriptional programs remain active in the absence of external cues. Understanding this interaction could provide new insights into the molecular regulation of lipid homeostasis and identify targets relevant to metabolic and skin-related disorders associated with impaired lipid regulation.

P033
Development of a senior canine skin model for NAM-based safety assessment and efficacy of novel pet cosmetic ingredients
ABSTRACT #319

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The rapid expansion of the pet cosmetics market has increased the need for scientifically robust and animal free safety assessment systems specifically tailored to canine physiology. Furthermore, most cosmetic ingredients are originally developed for humans and tested exclusively on human skin, so, there is a critical need to newly evaluate and identify ingredients that demonstrate true efficacy for pets. Although several canine cell–based artificial skin models have been developed to date, truly specialized and highly tailored models are still lacking. This study developed a senior canine skin model using verteporfin, a pharmacological inhibitor of Yes-associated protein (YAP), a key regulator of epidermal homeostasis and ECM deposition. The model enables evaluation of skin barrier integrity and the effects of pet grooming products, addressing the gap in research on senior canine skin. Canine epidermal keratinocyte progenitors (CPEK) were purchased from CELLnTEC Advanced Cell Systems (Switzerland). The cells were treated with verteporfin (0.1–10 μM) under non-photoactivated conditions. In addition, we developed a method for constructing an epidermal layer model using CPEK. This artificial skin model was treated with verteporfin to evaluate subsequent changes in skin barrier and ECM characteristics. The results showed that verteporfin significantly reduced YAP

nuclear localization and downregulated ECM components, confirming its anti-fibrotic potential. Interestingly, 24 hr treatment led to a transient decrease in desmosomal proteins, suggesting weakened intercellular adhesion. In addition, analysis of the skin model revealed a reduction in epidermal thickness and a decrease in claudin-1 levels, suggesting compromised barrier integrity. Finally, the compromised skin model was employed to screen candidate compounds with proven benefits for canine skin health. This model may contribute to regulatory acceptance and improved formulation strategies in the growing pet skincare sector. Further studies are needed to optimize the canine skin model and explore broader applications of pet grooming formulations.

P034
Permeability of hazardous agents in cooling lubricants after application of occupational skin protection products
ABSTRACT #370

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Skin barrier creams (BCs) are cosmetic products with the purpose of protecting skin from hazardous substances or water. In many occupations, employees are frequently in contact with liquids, cleaning agents, or chemicals that can damage the skin barrier. This leads to skin diseases, which are among the most common occupational diseases in Europe. As the use of gloves is not permitted during certain tasks for safety reasons, employers are often obligated to provide suitable skin protective agents. As the efficacy of BCs remains highly controversial in literature, the present work aimed to investigate the influence of two BCs on the permeation characteristics of formaldehyde (FA) and monoethanolamine (MEA), which are frequent contact allergens found in metalworking fluids (MWFs). Franz diffusion cells were used as the current gold standard method for testing in vitro penetration through the skin. An appropriate study protocol was established using ex vivo porcine skin to assess the substance-specific permeability with and without prior use of the two protective agents. The amount of permeated test substances was analyzed by spectroscopic methods. The study demonstrated that the prior application of both BCs significantly reduced FA permeation through the skin ($p = 0.0322$ and 0.0420, respectively). Additionally, it was shown that the use of only one of the BCs significantly reduced MEA permeation (from 5,84 % to 2,32 %, $p = 0,0318$), while the application of the second one did not affect the permeated amount (5,84 % vs. 5,80 %). These results underline the necessity for more in-depth investigations of BCs since manufacturers often only test one irritant but claim protection against a wide range of substances. However, further studies are needed to validate these results and to draw definitive conclusions about the efficacy of BCs with respect to individual selected hazardous working substances.

P035
Addressing toxicological data gaps in cosmetic safety assessment: A case study on the regulatory challenges related to the use of read across and NAMs
ABSTRACT #385

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Background: Due to the ban of animal testing in cosmetics and the limited availability of validated new approach methodologies (NAM) for complex systemic endpoints, filling toxicological data gaps for cosmetic ingredients remains a significant challenge. Read-across is a key technique to address these gaps. However, the Scientific Committee on Consumer Safety (SCCS) does not currently consider it sufficient as a standalone tool, and its use differs from our experiences in how read across is being applied in other EU regulatory frameworks (e.g., REACH and BPR). This poster presents a case study illustrating these considerations and the steps taken to address them. Methods and Results: In the case study, a comprehensive substance-specific dataset was developed, including guideline-compliant in vitro irritation, 99ensitization, genotoxicity

and dermal absorption studies. A structured read-across approach aligned with the ECHA Read-Across Assessment Framework (RAAF) was applied to address the systemic toxicity endpoints. To further reduce uncertainties in the read-across hypothesis, NAMs — including bioactivity assays (e.g., ToxProfiler®, ReproTracker®) — were generated for both the target and source substances. However, results were challenging to interpret in a regulatory context, highlighting the practical limitations of the available NAMs for complex systemic endpoints. Despite all the efforts to develop a scientifically sound dossier, the read across was not accepted. Conclusions: The poster highlights several regulatory challenges including (1) variations in the application of read-across for complex systemic endpoints between SCCS and some other EU regulatory frameworks (e.g., REACH and BPR); (2) selecting appropriate NAMs for complex endpoints and interpreting their results, and (3) the need for structured guidance on the regulatory use of NAM data and their relevance to the safety assessment of pharmacologically active cosmetics ingredients. In addition, the poster proposes the steps to be taken in order to address some of these challenges.

P036
Optical coherence tomography enhances mecanistic evaluation of ocular irritation in an ex vivo bovine eye model
ABSTRACT #420

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Background: Accurate assessment of ocular irritation is essential for the safety evaluation of pharmaceuticals and cosmetic ingredients. Historically, these assessments relied on the Draize rabbit eye test, increasingly questioned due to ethical concerns and limited human relevance. Although alternative methods such as the BCOP assay have reduced animal use, they mainly measure corneal opacity and permeability and provide limited information on layer-specific tissue responses. Objective: This study evaluated Optical Coherence Tomography (OCT) as a high-resolution imaging technique to characterize corneal responses to irritants in an ex vivo bovine eye model, aiming to improve mechanistic insight in non-animal ocular irritation testing. Methods: Fresh bovine eyes from a local abattoir were selected based on corneal clarity and integrity then were exposed to irritant and non-irritant substances: trichloroacetic acid (3% and 30%), ethanol (96%), benzalkonium chloride, cosmetic formulations, eye drops containing benzalkonium chloride, and saline controls. Structural corneal changes were monitored using spectral-domain OCT to measure central corneal thickness and visualize stromal architecture. Epithelial integrity was assessed using fluorescein staining under cobalt blue illumination. Results: OCT imaging revealed distinct structural signatures of ocular toxicity reflected by corneal thickness changes. The negative control (saline) induced negligible swelling (0.29%). Mild cosmetic products produced minimal responses (micellar water: 1.38%). Intermediate increases were observed with shampoo, EDTA and Tween 20 (2.73–3.60%) and with the tested ophthalmic drug (4.65%). Strong irritants produced pronounced stromal edema: trichloroacetic acid 3% increased corneal thickness by 17.34%, ethanol by 20.39%, and trichloroacetic acid 30% by 30.58%. Fluorescein staining revealed epithelial disruption after benzalkonium chloride exposure despite limited stromal swelling. Conclusion: Integrating OCT ocular irritation testing provides a quantitative, layer-resolved structural dimension to alternative toxicology methods. By revealing corneal injury patterns not detected by conventional opacity-based assays, OCT-supported models may strengthen the predictive performance of New Approach Methodologies (NAMs) for ocular safety assessment.

Computational toxicology – in silico modelling, read-across, artificial intelligence and machine learning

P037
Development of an in silico approach for predicting human internal exposure to topically applied cosmetic ingredients in NGRA
ABSTRACT #135

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In the development of cosmetics, the implementation of animal-free safety assessment, known as Next Generation Risk Assessment (NGRA), is indispensable. Internal exposure prediction using pharmacokinetic (PK) models is one of the key elements to achieve NGRA. However, for many cosmetic ingredients, applying such models in risk assessment is challenging due to the limited availability of in vivo PK data for model validation and to the difficulty of obtaining reliable input parameters from in vitro experiments. In this study, we focused on a compartment model that can directly calculate plasma concentrations from ADME parameters. By incorporating the predictive accuracy of each parameter into the input, we aimed to achieve conservative prediction of human plasma concentration without observed PK data based solely on in silico prediction. To conservatively estimate internal exposure during cosmetic use, the maximum steady-state plasma concentration (Css,max) was predicted. Css,max was predicted based on a two-compartment model with the following three parameters; the skin permeability coefficient (Kp), total clearance (CLtot), and volume of distribution (Vd). For these three parameters, experimental values were collected for 326, 1564, and 1389 compounds, respectively. Monte Carlo simulations were conducted considering the variability in prediction accuracy for each parameter, and the 95th percentile was adopted as a conservative estimate of plasma concentration. Exposure scenarios for both rinse-off and leave-on products were examined. Of 48 compounds with observed three PK parameters (Kp, CLtot, Vd) in human , 46 compounds provided equivalent or higher values of Css,max by in silico prediction than by the calculation from observed PK parameters. These results suggested the potential applicability of our in silico method for the internal exposure prediction of cosmetic ingredients in NGRA.

P038
Computational prediction of the kinetic and toxicological parameters of cylindrospermopsin

ABSTRACT #138
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Cylindrospermopsin (CYN) is a cyanobacterial toxin with hepatotoxic, genotoxic, and neurotoxic potential, posing a threat to public health. Next-Generation Risk Assessment (NGRA) methods are increasingly used to evaluate toxicological potency in early stages. This study assessed the kinetic and toxicological profiles of CYN and its potential metabolites in silico using the BioTransformer 3.0 tool. ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) prediction was performed through the ADMETlab platform. Results showed that both the parent toxin (CYN) and its predicted metabolites (M1–M9) display low intestinal permeability (Caco-2 cells). Regarding human intestinal absorption (HIA), results varied depending on the compound: very low for M3 (3.6%) and M4 (0.1%), but high for M7 (98.6%) and M9 (98.9%). The probability of CYN and its metabolites being metabolized by cytochrome P450 isoforms was low. Significant differences in plasma protein binding (PPB), half-life ($T_1/_2$), and plasma clearance (CLplasma) were found, suggesting distinct excretion pathways for CYN and its metabolites. All compounds showed a high probability of hepatotoxicity, genotoxicity, and human nephrotoxicity. However, variations were observed in mutagenicity (Ames test) and carcinogenicity, with some metabolites (M3, M4, and M9) exhibiting higher predicted probabilities than CYN. Metabolites undergoing major structural modifications of the uracil ring showed markedly reduced toxicity. These results highlight the importance of evaluating not only the parent toxin but also its metabolites to fully understand the toxicological risks of CYN exposure. Computational tools such as BioTransformer and ADMETlab provide a valuable preliminary approach to the kinetic-toxicological profile, supporting and guiding future experimental research. Acknowledgements: Project PID2023-147444OB-I00 funded by MICIU/AEI /10.13039/501100011033 and by FEDER, EU. VII PPTI-US 2025 (Use of General Research Services (I.3) and for Research and Knowledge Transfer Management (I.7)). Keywords: In

silico, Biotransformation, Predictive Toxicity, ADME.

P039
Preliminary machine learning approach to the immunotoxicity of cosmetic related PFAS
ABSTRACT #159

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Background: Some perfluoroalkyl substances (PFAS) used as cosmetics ingredients remain unregulated despite their toxic potential. Traditional testing is costly, and animal testing is being banned making machine learning (ML) an alternative for classifying and prioritizing potential immunotoxic substances. Objetives: Using machine learning algorithms to classify PFAS-related cosmetic ingredients according to their immunotoxic activity. Methods: A database was created using the U.S. EPA's CompTox data. Machine learning models were trained on 1,701 chemicals, including their physicochemical properties and molecular fingerprints. Model performance was evaluated, and SHAP analysis identified key molecular features influencing classification. Additionally, PFAS from the cosmetics ingredient list were classified using the selected model. Results: The best-performing model revealed that PFAS with cyclic structures are more likely to exhibit immunotoxic activity. Conclusion: ML models suggest that cyclic PFAS in the COSING database may have immunotoxic potential, but experimental validation is needed. ML is a cost-effective tool for prioritizing substances, guiding further testing on high-risk compounds.

P040
Evolution of AI-based toxicity prediction and the emerging role of third-generation architectures
ABSTRACT #173

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The development of AI-based toxicity prediction systems can be divided into three generations, each reflecting major milestones in artificial intelligence technology. The first generation was represented by rule-based expert systems such as Derek Nexus, which relied on manually encoded structural alerts and mechanistic knowledge. The second generation introduced data-driven approaches based on statistical learning and chemoinformatics, exemplified by tools such as the OECD QSAR Toolbox and EPI Suite. These systems improved prediction coverage and reproducibility but remained limited by the quality and scope of available datasets. The ongoing third generation marks a paradigm shift driven by the advent of deep learning and generative AI technologies, particularly those employing Attention and Transformer architectures. These architectures, originally developed in fields such as natural language processing, have demonstrated unprecedented capabilities in contextual learning and complex pattern recognition, as evidenced by ChatGPT and AlphaFold. Applying such architectures to toxicology, however, requires new strategies that differ fundamentally from the AI frameworks of previous generations. This poster discusses the conceptual foundations of Attention mechanisms and Transformer models and explores their potential to redefine the methodological basis of computational toxicology. By integrating these third-generation AI technologies, toxicological modeling may evolve from rule- or data-driven paradigms toward systems capable of adaptive reasoning, multi-modal integration, and generative hypothesis formation, thereby advancing the future of AI-driven toxicology research.

P041
EndocrineAI: an AI-driven platform for endocrine disruption screening
ABSTRACT #189

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Abstract Background: Endocrine-disrupting chemicals (EDCs) pose significant regulatory and public-health concerns. The U.S. EPA EDSP and OECD guidance emphasize replacing animal testing with validated new approach methodologies (NAMs). However, the lack of transparent, easy-to-use QSAR/ML systems limits practical adoption by toxicologists and regulators. Objectives: We

developed EndocrineAI, an interactive, AI-based cheminformatics platform that enables non-expert users to build, validate, and deploy quantitative structure–activity relationship (QSAR) models for endocrine disruption assessment. Materials and Methods: The tool was implemented in Python (3.11) using Streamlit for the user interface and integrates RDKit for molecular fingerprint generation and scikit-learn for machine-learning workflows. It supports Random Forest, SVM, k-NN, and MLP algorithms for both classification (EDC vs non-EDC) and regression (pIC_{50} prediction). Optional SMOTE balancing, GridSearchCV hyper-parameter tuning, and k-fold cross-validation ensure robust model performance. Real-time visualizations (ROC curves, confusion matrices, R^2 plots) and similarity-based analogue analysis enhance interpretability and regulatory transparency. Results: EndocrineAI achieved reproducible accuracies exceeding 0.85 for representative EDSP reference datasets across multiple receptor targets. All trained models, datasets, and logs are stored locally with timestamps, ensuring full auditability and compliance with OECD QSAR Principles. The modular design supports both auto-prediction (rapid screening) and interactive analysis modes for research or regulatory review. Conclusion: EndocrineAI exemplifies a next-generation NAM supporting the 3Rs (Replacement, Reduction, Refinement) principles by providing an accessible, validated, and extensible platform for endocrine disruption screening.

P042
Establishment of readiness criteria for AI-models for risk assessment: ReadyAI
ABSTRACT #200

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Recent advances in artificial intelligence (AI), covering 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 increasingly 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. To effectively and reliably integrate these models into regulatory frameworks, it is essential that regulatory authorities, policymakers, 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 ReadyAI. We aim to address the key challenges associated with

implementing AI in toxicology by developing a comprehensive scoring system for evaluating the development, training, and validation of AI-based models applied in chemical risk assessment, enabling a more independent and objective assessment of the regulatory applicability of AI-based models. 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 models applied in chemical risk assessment.

P043
Cheminformatics interaction profiles implicate the UV filter benzophenone-4 as a potential obesogen via PPARY activation
ABSTRACT #203

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Obesogens are chemicals that disrupt metabolic regulation and bias cell fate toward adiposity. We developed a mechanism-focused read-across workflow that compresses pose-level protein-ligand contacts from docking into a 327-feature interaction-profile fingerprint. After dimensionality reduction and unsupervised clustering of 6,022 Tox21 compounds, cluster-level enrichment against Tox21 assay calls mapped chemical neighborhoods to specific bioactivities. A cluster enriched for peroxisome proliferator-activated receptor gamma (PPARγ) agonism included the sunscreen agent benzophenone-4 (BP-4; sulisobenzone), despite its “inactive” annotation in Tox21. Orthogonal assays confirmed selective PPARγ binding by BP-4 and recruitment of SRC-2 and PGC-1α coactivator peptides. In human bone marrow-derived mesenchymal stem cells, BP-4 enhanced adipogenic differentiation, increased lipid accumulation, and elevated adiponectin production, indicating an adipogenic response in vitro. These findings nominate BP-4 as a candidate obesogen acting through PPARγ and illustrate how interaction-profile read-across can uncover overlooked bioactivity and prioritise environmental chemicals for mechanistic testing and safety evaluation.

P044
Building a quantitative adverse outcome pathway for AOP #38 – protein alkylation leads to liver fibrosis
ABSTRACT #212

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Background and objectives: Sustained exposure to chemicals may induce liver fibrosis, which in turn can develop into severe and irreversible adversities like cirrhosis or carcinoma. Understanding the mechanisms by which chemicals induce fibrosis is crucial for assessing their safety and implementing regulations to protect human health, yet there is limited quantitative understanding of how key-events (KEs) leading up to liver fibrosis are linked. Here, we used a novel gene expression-based approach to quantify KEs, and dynamical mathematical modeling to establish KE relationships (KERs) for adverse outcome pathway (AOP) #38 based on in vivo data. Material and Methods: We acquired toxicogenomic data from rat studies following either exposure to fibrosis inducing compounds, or surgically induced ischemia reperfusion injury (IRI). We applied weighted gene co-expression network analysis (WGCNA) in order to quantify the KEs defined in AOP#38 as we could trace back the joint transcriptional response of specific gene-sets to the respective biological events. We then modeled the relationships between KEs as ordinary differential equations (ODEs) and integrated them into a mechanistic model. Results Enrichment analysis of GO and pathway terms relevant to specific KEs, and quantitative comparison of pathology scores to dynamic toxicogenomic profiles along with tracking of marker genes, indicated that gene co-expression modules can robustly be matched to KEs. Dynamic modelling of those KEs showed that a quantitative AOP (qAOP) based purely on a linear sequence of events failed to capture the observed complex KE dynamics following chemical exposure.

Addition of feedback representing the balance between damage and repair improved the model considerably and provided a good fit to our time-course IRI data. Discussion and conclusion We conclude that ODE based modelling of toxicogenomic data can give valuable insight into KERs within an AOP. Furthermore, this highlights the importance of sufficiently detailed time-series data for qAOP development.

P046
Enhancing in silico prediction of Ames mutagenicity for organosilicon compounds and exploring chemical space boundaries
ABSTRACT #254

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Organosilicon chemistry, a subsection of organic chemistry with unique chemical properties, is typically underrepresented in the training sets of (Q)SAR models, and often outside the applicability domain. Using bacterial reverse mutation as an endpoint for a proof-of-concept study, we compiled a peer-reviewed reference dataset of >100 organosilicon substances and publicly available reliable Ames tests to assess the predictive performance of fourteen in silico methods, including expert systems, hybrid and statistical models with varying underlying algorithms (Derek Nexus, Sarah Nexus, VEGA mutagenicity models, TEST mutagenicity models, OECD QSAR Toolbox profilers). The assessment showed that most, but not all, tools predict mutagenic potential of organosilicon chemistry as accurately as of other organic datasets, with six tools providing balanced accuracies ≥ 80%. The nature of the model algorithm was identified as the key driver for accuracy. The presence of the silicon atom sometimes resulted in substances being outside model-specific applicability domains, but this did not necessarily correlate with model predictive performance Furthermore, the assessment showed that silicon-based chemistry does not possess intrinsic gene mutation potential. All positive Ames test results in the dataset could be linked to organofunctional structural alerts known to be related to mutagenicity.

P047
Systematic linkage of transcriptomics data to adverse outcome pathways for toxicity prediction
ABSTRACT #280

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Adverse Outcome Pathways (AOPs) are structured frameworks that describe how chemical exposure can lead to adverse health effects via so-called Key Events (KEs). To improve their use in risk assessment, we explored how transcriptomics data can be systematically linked to AOPs. Our goal was to enrich AOPs with genesets to better connect molecular changes to toxicological outcomes. Based on the AOP-Wiki, we generated extensive AOP networks specific to liver, brain, kidney, and lung toxicity. For KEs lacking ontological annotations, we systematically mapped them to biological process terms from Gene Ontology (GO) using AMiGO2, and then linked these to corresponding genesets. We tested the approach by using a publicly available Open TG-GATES human liver dataset exposed to food additives or pesticides. This dataset was used to quantify KE activity within the liver AOP network by following the single-sample gene set enrichment analysis (ssGSEA) approach. The AOP networks for liver, brain, kidney, and lung comprise 195, 222, 68, and 142 KEs, respectively, of which 56.7% were already annotated in total. We added annotations for the remaining unannotated ones, and tested our approach on the liver AOP network, resulting in 76% of KEs linked to genesets. Using these KE genesets, we calculated KE activity scores as a result of exposure to our compounds of interest. This resulted in the prediction of significantly active

KEs within the liver network. Notably, food additives primarily activated MIEs at the early time point when tested at high concentration, suggesting early molecular perturbations that may trigger downstream toxicological responses. Despite remaining challenges such as incomplete annotation and manual curation requirements, this work demonstrates a systematic strategy for integrating transcriptomics with AOP frameworks. By linking KEs to functional genesets and calculating their activity in response to chemical exposure, this research allows for more mechanistic, data-driven chemical risk assessments.

P048
AI4AOPOMICS: artificial intelligence for integrating omics data into adverse outcome pathways
ABSTRACT #283

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With the advancement of sequencing technologies, omics—which capture the collective molecular profiles of biological systems such as genes, proteins, and metabolites—have been actively applied in the field of toxicology. In parallel, there has been a paradigm shift toward the 3Rs (Replacement, Reduction, and Refinement) in animal testing, which have traditionally been the gold standard of toxicity assessment. Consequently, omics data are now recognized as one of the most promising new approach methodologies (NAMs) for modernizing chemical risk assessment within a regulatory context. One strategy to leverage omics data in chemical risk assessment is to integrate them into the adverse outcome pathway (AOP) framework. An AOP is a conceptual framework that describes a series of causally linked biological events at different levels of organization (e.g., cellular, tissue, organ) leading to an adverse health effect in an organism following exposure to a stressor such as a chemical. Because omics approaches are inherently data-driven and non-targeted, they generate comprehensive molecular profiles that reflect system-wide responses to chemical exposure. When integrated with the knowledge-based and structured AOP framework, omics data can be filtered and interpreted in a biologically meaningful way, while also enriching and expanding existing AOPs by revealing novel mechanistic insights. Thus, omics and AOPs can be used in a complementary manner to enhance mechanistic understanding and improve the predictive power of chemical risk assessment. In this study, we developed a practical pipeline to support the integration of omics data into the AOP framework and demonstrated its feasibility through a proof-of-concept case study. The pipeline consists of three steps: (1) omics data analysis, (2) mapping of omics data to AOP ontology, and (3) AOP confirmation through literature analysis. In conclusion, this study provides a practical framework for integrating omics data within the regulatory context, ultimately supporting the next generation of chemical risk assessment.

P049
Validation of AI-based toxicity prediction models with reference chemicals for regulatory applications
ABSTRACT #285

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The growing demand for animal-free safety assessment has accelerated the development of new approach methodologies (NAMs) that align with the principles of the 3Rs—Replacement, Reduction, and Refinement of animal use. Among various NAMs, artificial intelligence (AI)–based toxicity prediction models have emerged as promising tools to support regulatory toxicology by providing rapid, cost-effective, and mechanistically relevant hazard predictions. However, ensuring their scientific validity and regulatory acceptability remains a critical challenge. In our previous work, we developed comprehensive AI-based toxicity prediction models trained on

regulatory toxicity endpoints, including acute, chronic, and CMR (carcinogenicity, mutagenicity, reproductive toxicity) data [1-2]. These models were designed to integrate chemical structure information and endpoint-specific toxicological knowledge to enhance predictive reliability. To further explore their regulatory applicability, the present study focuses on the validation of these models using well-characterized reference chemicals compiled by international organizations such as ICCVAM and EURL. The use of reference chemicals provides an objective basis for assessing the models' predictive performance, consistency, and mechanistic relevance across different toxicity domains. Multiple validation case studies were conducted to examine model behavior against curated experimental data and to identify areas requiring further refinement, such as data representativeness and endpoint coverage. This work contributes to the ongoing discussion on the validation of AI-based NAMs in regulatory contexts and provides insights into how such models can complement existing safety assessment frameworks toward more ethical, efficient, and science-based decision-making in chemical risk assessment.

P050
Decoding biological processes through multi-omic network diffusion
ABSTRACT #290

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Motivation: Traditional approaches to investigating biological processes often lack tissue specificity and fail to integrate the complexity and diversity of biological modes of action. Drug-induced liver injury (DILI) remains a critical challenge in drug development and is broadly studied by the drug sector, providing a wealth of data including multi-omics' data whose richness appears of great interest. The data was leveraged in this study to illustrate how the network diffusion approach could help elucidate molecular mechanisms underlying complex processes leading to adverse effects. Results: We constructed a liver-specific, multi-layer molecular network integrating transcriptomics, proteomics, pathways, drugs and side-effects data and provide a diffusion-based analytical framework to highlight key mechanisms from toxicogenomics data. Using reference chemicals with well documented mechanism of actions as case studies, our approach revealed distinct adverse mechanisms, such as fatty acid metabolism disruptions, and identified biologically relevant subnetworks. Compared to traditional enrichment methods like ORA and GSEA, the diffusion-based framework demonstrated complementarity by identifying unique pathways and offering mechanistic insights. Concordance with literature and clinical data were evaluated and confirmed. Relevance: Considering the current regulatory context and the cosmetic industry's commitment to its values in developing innovative New Approach Methodologies (NAMs), it appears necessary to design in vitro human relevant models that addresses both scientific and industrial challenges. Scientific through exhaustive biological diversity and complexity coverage, and Industrial by developing regulatory accepted approaches ensuring consumer protection. Perspectives: Present work paves the way formultiple applications to widely decipher complex mechanisms of action, chemical biological activity for both adversity and efficacy purposes.

P051
Chemical clustering of natural complex substance (NCS) materials: A hierarchical clustering approach for use in non-animal safety assessments
ABSTRACT #294

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Background and Objectives: Clustering of natural complex substances (NCS) is a valuable approach for rapid data analysis, identification of read-across analogs, and establishing an efficient safety assessment process. However, this process is complicated by the various components with differing features that comprise individual NCS materials. To address this, the Research Institute for Fragrance Materials (RIFM) has selected 166 NCS materials, encompassing over 20 families and over 30 genera, to evaluate for clustering. Materials and Methods: Agglomerative hierarchical clustering (AHC) was used to create a hierarchy of NCS clusters. AHC is a bottom-up approach that treats each material as an individual cluster and then iteratively merges them until all materials are in a single cluster. Techniques such as Calinski-Harabasz index, elbow method, and silhouette score were used to determine the appropriate number of clusters. Results: Evaluation of the 166 NCS materials using AHC show that these materials cannot simply be clustered by genus. Therefore, understanding the compositions of these materials is required to determine a clear rationale for the formation of appropriate clusters. Additionally, when the composition was set to a specific threshold (such as 80%, 85%, or 90%) of the total composition, there were minimal to no changes to the clusters formed by AHC. Discussion and Conclusion: These methodologies allow for the formation of NCS clusters with similar toxicological properties. The clusters are primarily based on shared components and not genus. Furthermore, the threshold clustering shows that there is an insignificant change in the clusters when the lowest percentage components are removed. Therefore, toxicological profiles could be determined for NCS materials if the percentage of components meets the clustering threshold. Additionally, the clustering approach could be used as a basis for filling data gaps through read-across in NCS safety assessment due to the toxicological similarity of the clusters.

P052
Advancing chemical grouping: development and application of signature-based structure-activity groups for non-animal safety assessments
ABSTRACT #295

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Background and Objectives: The Research Institute for Fragrance Materials, Inc. (RIFM) has developed a robust, reliable, reproducible method for clustering chemicals based on their structural signatures and deriving structure-activity groups. This method facilitates the institutionalization of knowledge gained from manually assessing thousands of chemical pairings of fragrance ingredients. The technique improves accuracy, consistency, transparency, and explainability for evaluating chemical safety while reducing reliance on expert judgment and any associated bias. Materials and Methods: A material's signature-based structure-activity group is created via a top-down approach using standardized signature trees based on Indicator Phrases (IPs) representing seminal sub-structural features. We have applied the approach to over 6,000 discrete fragrances and fragrance-like organic chemicals (e.g. organic compounds of the chemical classes described in the inventory such as aldehyde, ketone, esters, etc.), and it has been shown to perform well for various

properties and parameters observed in this chemical space. The signature trees are adaptable and can be expanded for IPs not found in fragrance materials. Results: The structure-activity groups readily allow for transparent and repeatable separation of an inventory of thousands of chemicals into clusters of chemicals that share the same IPs. Adjacent groups that share all but one or two of the same IPs can be identified, thereby effortlessly expanding the range of potential read-across source substances. Discussion and Conclusion: With its ease of interpretation, the system facilitates discussions among scientists with different levels of chemical knowledge. In addition to clustering for data-gap filling through read-across, other applications include prioritization for testing and predictive toxicology by encoding IPs using various machine-learning techniques.

P053
An approach to characterise and define tolerable uncertainty in read-across for toxicity prediction
ABSTRACT #316

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Read-across is a widely applied in silico new approach methodology (NAM) to fill toxicological data gaps. Frameworks for read-across are formalised into workflows by various regulatory agencies with associated guidance from e.g. ECHA, EFSA, OECD. The consideration of uncertainty is a key component of the assessment of the robustness of a read-across, with tolerable uncertainty being defined in the problem formulation. The aim of this investigation was to identify the key uncertainties in a read-across that can be applied to define and assess tolerable overall uncertainty. This work builds on the previous analysis of read-across uncertainty (Schultz et al., 2019) and applies published guidance on uncertainty assessment (EFSA, 2018). Seven key areas of uncertainty in read-across were identified and schemes to quantify them into very low, low, moderate or high uncertainty are provided. The seven areas of uncertainty are: chemical similarity and class; functional groups and / or molecular substructures for toxicodynamics, toxicokinetics and formation of common metabolites; physico-chemical properties relating to toxicokinetics; and data quality. The relative impacts of each of the seven areas of uncertainty on a particular endpoint can be adjusted. Thus, toxicokinetics will have a high impact on uncertainty for repeated dose effects of low / no toxicity compounds (assuming clearance is a key factor and no specific mechanism of action). These areas of high impact uncertainty will vary according to endpoint, substance and use case. It is the areas of high impact uncertainty that drive the overall uncertainty, with the overall uncertainty being equivalent to the greatest individual area of high impact uncertainty. This process provides a means to rapidly assess overall tolerable uncertainty. Examples from published read-across demonstrate its flexibility and adaptability.

P054
Heme metabolism and mycotoxins: lessons learned from Atlantic salmon - a computational multi-target investigation
ABSTRACT #325

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Mycotoxins, widespread contaminants, raise food safety concerns. Among these, enniatin B (ENNB) and beauvericin (BEA) are detected in food and feed and, although their effects in humans are still debated, their toxicity is evident in some animals, including Atlantic salmon where they cause impaired growth and anemia-like phenotype [1]. While these effects are increasingly documented in fishes, with growing evidence pointing to impairment of heme biosynthesis, the relevance to humans remains unclear and the possible underlying mechanisms are largely undefined. Importantly, heme biosynthesis and transport involve several evolutionarily conserved proteins, and even moderate perturbations may translate into altered heme availability. The observation of anemia-like phenotype in salmon therefore raises the question of whether analogous mechanisms may be relevant to humans. This study, part of the MYTOXA project (Norwegian

Research Council, grant number 34401), presents a computational multi-target NAMs-based strategy to evaluate whether ENNB and BEA can plausibly interact with salmon proteins involved in heme biosynthesis, including CPOX, FECH, HMBS, PPOX, UROD, and UROS, and transport, such as FLVCR1, FLVCR2, HMOX1, and SLC46A1. Using Boltz-2 [2], an AI-based 3D protein modelling tool, followed by molecular dynamics simulations, binding plausibility and conformational stability were assessed. Building on these results, the interactions identified in salmon are being translated to the corresponding human orthologues, also integrating in vitro investigations, to explore whether the molecular mechanisms observable in fish may also be relevant for humans. In vitro assays on human and fish cells (e.g. HepG2 and SOB-15) described significant perturbations of heme precursors (e.g. protoporphyrin IX) suggesting meaningful mode of actions to investigate further in humans. Overall, this work frames Atlantic salmon as a valuable biological sentinel and molecular leverage point to characterize the risks for humans, supports a mechanism-driven interpretation of emerging mycotoxin risks, and provides a testable road-map to guide follow-up experimental studies.

P055
Guardrailed, auditable agentic workflows for computational toxicology via the model context protocol (MCP)
ABSTRACT #336

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Computational toxicology increasingly combines heterogeneous evidence streams—high-throughput screening, exposure and use data, (Q)SAR and read-across, adverse outcome pathways (AOPs), and physiologically based kinetic (PBK/PBPK) models—within integrated approaches to testing and assessment (IATA). Yet practical evidence pipelines remain fragmented across dashboards, GUI-driven tools, incompatible data formats, and licensing boundaries. This fragmentation slows NAM-based assessment and makes computational results difficult to audit, reproduce, and govern. Large language models can lower the interface barrier, but free-text interaction alone is insufficient for regulatory-grade science. We present ToxMCP, a federated suite of Model Context Protocol (MCP) servers that exposes established toxicology capabilities as typed tools with explicit input/output schemas, provenance bundles, and enforceable policy hooks (identity normalization, applicability-domain checks, and "critical-action" confirmation for state-changing or high-cost runs). The server suite integrates chemical identity and regulatory context retrieval (EPA CompTox/DSSTox), rapid property and ADMET profiling (ADMETlab 3.0), mechanistic pathway retrieval and structuring (AOP knowledge services), OECD QSAR Toolbox workflows for profiling and read-across, and mechanistic PBPK simulation via the Open Systems Pharmacology Suite. In exemplar multi-tool workflows, natural-language risk questions are compiled into auditable tool-call traces that return interpretable outputs such as tissue AUC/Cmax/Tmax, sensitivity analyses, analogue evidence matrices, and dossier-ready artifacts, together with machine-verifiable metadata on tool versions, parameters, and data sources. We further outline an evaluation protocol for workflow validity, computational reproducibility, scientific compliance, and efficiency. ToxMCP provides an infrastructure blueprint for accountable agentic workflows in computational toxicology, accelerating multi-tool analysis while preserving the evidentiary structure required for peer review and regulatory-adjacent decision support.

P056
O-QT: auditable AI-assisted QSAR toolbox read-across workflows for VHP4Safety implementation
ABSTRACT #339

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Read-across is a cornerstone of non-animal hazard assessment, but practical implementation still depends heavily on manual, GUI-driven workflows in the OECD QSAR Toolbox. This creates bottlenecks for

transparent documentation (analogue selection rationale, applicability domain, and uncertainty communication) and limits integration of read-across outputs into broader digital evidence pipelines such as the Virtual Human Platform for Safety Assessment (VHP4Safety), which aims to support animal-free safety testing by integrating human-relevant tools, models, and data. We present O-QT, an auditable, tool-driven AI workflow that orchestrates OECD QSAR Toolbox WebAPI functions for profiling, analogue identification, and quantitative read-across, while producing reviewer-friendly artifacts. O-QT wraps Toolbox operations as typed actions with explicit parameters, structured outputs, and policy checks (e.g., mandatory chemical identity normalization, reproducible selection filters, and applicability-domain gating). Each workflow yields (i) a machine-readable trace of all Toolbox API calls and intermediate results (JSONL), (ii) exported evidence matrices supporting analogue selection and endpoint predictions, and (iii) a dossier-style PDF report aligned with QSAR Toolbox outputs. Within VHP4Safety, O-QT is deployed as a reusable service to strengthen computational hazard evidence and enable downstream coupling to mechanistic and toxicokinetic modules. We demonstrate end-to-end workflows from a SMILES query to a complete read-across dossier and highlight how structured logs and exported artifacts improve traceability compared with conversational LLM use. O-QT reduces friction in read-across while preserving transparency requirements, supporting scalable NAM evidence generation and interoperable safety assessment workflows.

P057
Macrophage-explicit PBPK model predicts long-term human organ accumulation of ingested microplastics
ABSTRACT #354

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Background and Objectives Environmental contamination by nano- and microplastics (NMPs) raises concerns about human health from chronic dietary exposure. However, high-dose, short-term studies hinder human risk assessment translation. We developed a macrophage-explicit physiologically based pharmacokinetic (PBPK) model to predict long-term organ accumulation and identify biological drivers of internal dose metrics over 10 years. Material and Methods The model describes microplastics (MPs) kinetics across blood and organ-specific macrophage pools. Daily intake was 583 ng/day [1]. Systemic bioavailability and rate constants for MPs uptake into monocytes and tissue macrophage pools were calibrated using mouse organ-burden data [2]. High fitted bioavailability, potentially overestimating source data, was retained for consistency. Risk-thresholds were established by converting intracellular particle counts from macrophage studies to mass-based concentrations (assuming 20 μm diameter) and scaling via cytotoxicity benchmarks [3]. Post-10-year trends were linearly extrapolated to estimate threshold arrival times. Results After one year, direct blood-to-tissue macrophage translocation dominated accumulation across all organs, while macrophage lifespan influence was minimal. In the spleen, the monocyte-mediated pathway re-emerged as a significant secondary factor after four years, reflecting high macrophage density. Our simulations suggest that reaching risk-threshold concentrations under current exposure levels would likely require a timeframe of several centuries or longer. Discussion and Conclusion Long-term MPs accumulation is governed by direct translocation into tissue macrophages, representing factors such as vascular endothelial permeability and capture efficiency. In macrophage-rich tissues, infiltration via monocytes is a relevant risk factor. These findings suggest current environmental exposures require centuries to reach toxicity thresholds, providing a quantitative roadmap for refining human risk estimates in next-generation risk assessment (NGRA).

P058
Automated quantification of collagen gel contraction using a fine-tuned foundation model for in vitro toxicity assessment
ABSTRACT #375

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Wound healing following lung injury is a critical biological process in which appropriate tissue repair determines progression to fibrosis. Fibroblast-mediated extracellular matrix contraction plays a central role in tissue remodeling, and the collagen gel contraction assay is widely used to evaluate this activity. However, conventional quantification relies on manual boundary tracing, which is time-consuming and susceptible to inter-operator variability, limiting reproducibility. In this study, we developed an automated and reproducible segmentation framework for collagen gel area quantification using a fine-tuned foundation segmentation model. Human lung fibroblasts (MRC-5) were cultured under control conditions, stimulated with TGF-β1, or exposed to chemicals with potential inhalation toxicity. Gel images were acquired at 0, 24, and 48 hours. The training dataset consisted of manually annotated gel images collected over multiple years under diverse experimental conditions. Representative samples encompassing baseline, fibrotic stimulation, and toxicant-induced contraction phenotypes were curated and annotated by an experienced domain expert to capture broad morphological variability and experimental heterogeneity. The image encoder backbone of SAM3 was fine-tuned to adapt feature representations to gel-specific structural and textural characteristics. The resulting model enabled automated gel segmentation without additional preprocessing or parameter tuning. Segmentation performance was evaluated by comparing predicted masks with ground-truth annotations using Intersection over Union (IoU). The fine-tuned model demonstrated consistently high concordance with manual measurements across all conditions and time points, indicating robust and operator-independent quantification. This backbone adaptation strategy enables scalable, reproducible, and high-throughput analysis of collagen gel contraction assays. The framework provides a practical foundation for quantitative fibrosis and wound repair research, supporting large-scale screening and downstream data-driven modeling approaches.

P059
Case study on the use of AOP-based AI model for prioritization of endocrine disrupting chemicals from consumer products
ABSTRACT #379

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This case study aimed to demonstrate an Integrated Approach to Testing and Assessment (IATA) framework that leverages adverse outcome pathway (AOP) anchoring and explainable artificial intelligence (AI) to support prioritization of potential endocrine-disrupting chemicals (EDCs) in consumer products. The approach is designed to provide a transparent, mechanistically interpretable workflow that integrates hazard prediction with exposure considerations while remaining scalable for screening large chemical inventories. In the model development component, AI-based prediction models are built from available toxicological evidence, including new approach methodologies (NAMs) such as in vitro bioactivity data and relevant in vivo endpoints, and model outputs are linked to biological key events across the AOP continuum from molecular initiating events through intermediate key events to adverse outcomes. To enhance interpretability and decision utility, the framework applies a tiered weighting concept that reflects the position of each key event within the AOP, and integrates these weighted outputs through structured decision rules to generate an overall hazard indicator for each chemical. In the application component, the hazard indicator is combined with an exposure-informed selection strategy using multiple lines of evidence, such as patterns of chemical use in consumer products, indicators of potential for human contact, and

available biomonitoring or population-level exposure information, thereby enabling a weight-of-evidence evaluation that distinguishes chemicals by relative level of concern. Final outputs can be summarized in a risk classification matrix that jointly considers exposure and hazard signals and assigns chemicals to high, medium, or low priority categories, where high priority supports comprehensive assessment, medium priority supports targeted data generation to resolve uncertainties, and low priority supports routine monitoring. Overall, this AOP-anchored, explainable AI IATA illustrates a practical and adaptable concept for mechanistically informed, data-driven chemical prioritization that can be tailored to different jurisdictions and regulatory contexts, supporting both hazard-based classification.

P060
A hybrid in silico/in vitro approach to address applicability-domain limitations in chemical carcinogenicity prediction
ABSTRACT #381

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Background and Objectives: In silico approaches, ranging from QSAR and read-across to machine-learning models, enable rapid, low-cost screening for genotoxicity and carcinogenicity based on structure–activity relationships, supporting hazard prioritization. However, predictive reliability is constrained by the applicability domain (AD). When models are used beyond the chemistries represented in the training data, uncertainty increases and novel or underrepresented compounds are prone to misclassification [1]. In this work, in silico and in vitro tools were integrated to mitigate the AD limitation. Materials and Methods: A multitask graph neural network (MT-GNN) was developed to predict mouse carcinogenicity, using auxiliary tasks based on carcinogenicity-relevant in vitro bioassays and embeddings derived from stimulus-induced gene expression. Engineered graph-level features were introduced to encode key in vitro assay measurements related to carcinogenesis. These experimental inputs were combined with molecular structure to improve characterization of novel chemotypes. The AD was defined by confidence estimation [2] using a 0.5 ± 0.1 boundary: predictions in 0.4–0.6 were treated as out-zone, and those outside this range as in-zone. The test set comprised 18 carcinogens and 10 non-carcinogens. The single-task mouse carcinogenicity GNN (ST-GNN) model served as the baseline. Results: The baseline model yielded a ROC AUC of 0.7 and an MCC of 0.36, whereas MT-GNN achieved 0.78 and 0.38, respectively. In the baseline model, 10 of 28 test compounds lay outside the AD; after integrating bioassay-derived and gene expression features, 5 of these 10 fell within the AD. Discussion and Conclusion: The hybrid in silico/in vitro approach mitigated AD limitations of standard ST-GNN. Coupled with an in vitro screening platform aligned with selected bioassays and gene expression data, this strategy may represent an approach for identifying carcinogenic risk across broader chemical space while reducing dependence on AD constraints. Funding: This work was supported by the Science Committee of RA (#23LCG-1F002).

P061
Co-regulated gene network analysis to uncover mechanisms of liver toxicity liabilities in vitro
ABSTRACT #394

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Background and Purpose: Next-generation chemical risk assessment requires mechanism-based approaches that incorporate human-relevant new approach methodologies (NAMs) to better protect human health and meet evolving regulatory demands. High-throughput transcriptomics offers a powerful platform to detect early biological responses to chemical exposure in human in vitro systems. However, challenges remain in interpreting transcriptomic data and understanding the consistency of responses across test systems. Given the liver's central role in chemical toxicity, this study aims to improve liver toxicity prediction using co-expression network analysis across multiple

in vitro models. These networks support biological interpretation of transcriptomic perturbation and allow assessment of response concordance, aiding in defining system-specific applicability domains. Methods: Toxicogenomics datasets were generated using HepG2 cultured in amino acid-rich medium (AAGLY-HepG2), HepaRG, and primary human hepatocytes (PHH). Based on the previously established PHH TXG-MAPr [1], a compound set was selected to ensure robust network construction, comprising 39 hepatotoxic compounds, 8 pathway-specific reference compounds, and additional growth factors and cytokines. Compounds were tested at three concentrations, with samples collected at 8 and 24 hours for TempO-Seq. Differential expression analysis (DESeq2) was followed by Weighted Gene Correlation Network Analysis (WGCNA), and module preservation was assessed using Z-summary statistics and compared with existing TXG-MAPr datasets. Results: Approximately 75% of modules were preserved between PHH datasets. Key preserved modules were associated with stress responses, including unfolded protein response and inflammation. Around 80% module preservation was observed between HepG2 and AAGLY-HepG2. HepaRG cells showed the highest similarity to PHH, followed by AAGLY-HepG2, HepG2, and hiPSC-derived hepatocyte-like cells. Conserved responses across systems included unfolded protein response, heat shock, and oxidative stress pathways. Conclusions: WGCNA-based analysis captures conserved transcriptomic responses to chemical exposure across liver models. This approach supports identification of shared stress-response mechanisms and helps define the applicability of different in vitro systems for liver toxicity assessment.

P062
Using AI tools for systematic literature search, appraisal, and data extraction for the development of adverse outcome pathways
ABSTRACT #412

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Artificial intelligence (AI)–assisted tools can support multiple stages of evidence synthesis for toxicity assessment and accelerate the development of putative adverse outcome pathways (AOPs) aligned with OECD guidance. Here, we present an expert-in-the-loop workflow for developmental neurotoxicity (DNT), using decreased radial glia proliferation as an exemplar key event within a mechanistic mode-of-action framework. The first stage is a systematic review, beginning with developing a search strategy. The R package litsearchR was applied to a seed set of titles and abstracts to extract candidate terms and generate keyword co-occurrence networks, which were used to semi-automate search term refinement and Boolean query construction. This approach reduced reliance on investigator intuition and improved the transparency and reproducibility of search strategies. The retrieved articles' metadata were imported into RevTools for AI-supported screening, where relevance ranking, visualization, and de-duplication facilitated efficient prioritization during title/abstract and full-text selection, while preserving reviewer control over inclusion criteria. Study appraisal was structured using SciRAP, enabling standardized risk-of-bias and reporting quality judgments and improving consistency in evidence documentation across heterogeneous toxicology studies. Further, the data extraction approach used a large language model–based assistant (NotebookLM) to semi-automatically extract study characteristics, exposure metrics, outcomes, and effect estimates, with all extracted fields validated by human reviewers. The curated and appraised evidence base was then mined for mechanistic content to inform the development of OECD-compliant AOPs. Across five relevant studies, AI-assisted literature mapping organized evidence on upstream signal transduction perturbations and downstream consequences, including premature neurogenesis, increased astrocyte differentiation, neuroinflammation, and cognitive deficits. This workflow supported weight-of-evidence evaluation of key event relationships and helped prioritize targeted experimental studies to strengthen AOP confidence.

P063
Integrated analysis of cellular pathways of respiratory nanotoxicity

ABSTRACT #430

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Nanomaterials (NMs) are widely applied but raise concerns about respiratory toxicity with potential contributions to chronic lung diseases. While more than 50 transcriptomic datasets describing pulmonary responses to NMs are publicly available, most rely on bulk microarray or RNA sequencing that lack cellular resolution. Our recent single-cell transcriptomic mouse study revealed cell- and material-specific pulmonary perturbations with distinct inflammatory responses induced by different NMs: spherical and soot-like carbon nanoparticles (CNP), flexible double-walled carbon nanotubes (DWCNT), and rigid multi-walled carbon nanotubes (MWCNT). However, single-cell analysis is costly and currently available only for a limited number of materials. This study therefore aims to identify cell-type-specific responses to a broader coverage of nanomaterials by integrating bulk transcriptomic datasets with scRNA-seq references. We collected publicly available transcriptomic datasets related to carbon-based NM-induced respiratory toxicity. Deconvolution methods (CIBERSORTx, MuSiC, BayesPrism, InstaPrism etc.) were applied to estimate cell-type proportions and infer cell-type-specific gene expression patterns across different NM exposure conditions. Preliminary analyses revealed material-dependent alterations in lung cell-type composition, particularly involving epithelial cells and other cell types associated with early inflammatory responses. Ongoing analyses aim to identify gene pattern linked to NM-induced pulmonary toxicity and cellular response circuits triggered by nanomaterials. Benchmarking several deconvolution methods revealed inconsistent results, highlighting the lack of robust validation metrics for methods comparison. The reliability of deconvolution results were also influenced by the choice of the scRNAseq reference data and the specificity of marker genes. Additionally, certain methods require substantial computational power, which may limit their wider application. Our research presents a first step in leveraging existing bulk transcriptomic data with scarce single-cell sequencing data to reveal cell-specific mechanisms of respiratory nanotoxicity. This approach will improve mechanistic understanding for a wider panel of nanomaterials and may support in vitro models to recapitulate, thereby contribute to the safe-by-design strategy for future nanomaterials.

P318
Toxicoinformatics of molecular interactions for deciphering a plausible role of Bisphenol A in attention deficit hyperactivity disorder
ABSTRACT #452

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Exposure to bisphenol A (BPA), an endocrine disruptor at the dose below the acceptable daily intake threshold of 50 µg/kg/day, have been associated with neurological impairment related to Attention deficit hyperactivity disorder (ADHD). However, role of molecular interactions of various proteins which have role in ADHD with BPA are poorly known. The aim of the present study is to identify the contribution of molecular interaction of BPA and its metabolites to etiological/pathophysiology of ADHD through network toxicology study. Docking scores of BPA and its metabolites with selected proteins involved in the pathophysiology of ADHD were compared with their respective recognized inhibitors. The results showed greater inhibitory ability of BPA and its metabolites in comparison to respective known inhibitors of those enzymes. The promising docking scores of the proteins of protein-protein interaction (PPI) networks of selected proteins involved in ADHD pathophysiology compared to their respective inhibitors have also showed contribution of BPA to ADHD pathophysiology through PPI network. Interestingly, many close interactions between BPA and specific proteins were found through molecular docking including Van der Waals, hydrophobic, polar, and H-bonding interactions. Kyoto Encyclopedia of Genes and Genomes

(KEGG) pathway analysis, Gene Ontology (GO) and other enrichments exhibited the potential of BPA and its metabolites to contribute to ADHD pathophysiology through selected proteins of PPI network. The interaction of BPA and its metabolites with these selected proteins and their network proteins indicating the multiple entry point for BPA and its metabolites contributing to ADHD pathophysiology.

P320
Structure-based identification of potential small-molecule modulators of botulinum neurotoxin type a light chain ABSTRACT #461

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Botulinum neurotoxin type A (BoNT/A) is among the most potent biological toxins known, exerting its effect through a zinc-dependent metalloprotease light chain that cleaves neuronal SNARE proteins, leading to flaccid paralysis. Despite the availability of antitoxin therapies, effective strategies targeting intracellular toxin activity remain limited, highlighting the need for novel therapeutic approaches. In this study, a structure-based computational strategy was employed to explore potential small-molecule modulators of the BoNT/A light chain. Using publicly available structural data, key features of the catalytic domain and adjacent binding regions were analyzed to identify sites amenable to ligand interaction. A virtual screening workflow was applied to a focused compound library, with prioritization based on predicted binding affinity, structural complementarity, and physicochemical properties. Preliminary in silico analyses identified several candidate molecules demonstrating favorable predicted interactions within the active site and surrounding regions of the enzyme. These compounds exhibited binding profiles consistent with potential modulation of enzymatic activity. Comparative structural analysis also highlighted conserved features that may support future optimization and rational design efforts. These findings, while preliminary, demonstrate the utility of computational approaches as alternative methods for early-stage toxicological and pharmacological screening. Ongoing work aims to refine candidate selection and support subsequent experimental validation using in vitro systems. Keywords: Botulinum neurotoxin, Structure-based drug design, Virtual screening, Computational toxicology, Small-molecule modulators, New Approach Methodologies

P321
IN SILICO EVALUATION OF ANDROGEN RECEPTOR MODULATION OF BISPHENOL A SUBSTITUTES ABSTRACT #466

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Bisphenol A (BPA) is a known endocrine disrupting chemical. Concerns about its effects on human health and the environment have led to regulatory changes and the replacement with BPA substitutes, which are either bisphenol analogues or structurally different substances with similar properties. However, their toxicity and endocrine disrupting potential remain insufficiently studied. We focused our research on the androgen receptor (AR) modulation of BPA and 19 bisphenol substitutes using various in silico models: Endocrine Disruptome, VirtualToxLab, SwissTargetPrediction, and VEGA QSAR. The first three are based on binding to the AR, while the last is based on structure-activity relationship. Endocrine Disruptome predicts binding affinity for both agonist and antagonist AR conformations; VirtualToxLab estimates toxic potential and IC50; SwissTargetPrediction evaluates the probability of a substance targeting the AR; and VEGA QSAR predicts binding activity based on structure. Therefore, the predictions are diverse and provide an overview of the substances' potential for binding and (anti)androgenic effects. After comparing all predictions, we observed that most BPA substitutes that are not structurally similar to BPA show little or no potential for binding to and modulating the AR. In contrast, BPA analogues appear to have lower predicted binding affinities than BPA but higher predicted toxicity (lower IC50) and

potential for AR modulation. The exception is BPS and its derivatives, which show slightly lower binding affinities than BPA, while their potential for effects on the AR is practically non-existent. Our results suggest that in silico toxicology models can be used as an initial step in assessing toxicity and endocrine disruption potential. However, additional testing is required to further evaluate the AR modulation of bisphenol substitutes. Acknowledgements: This work was supported by the Slovenian Research Agency project N1-0363 and by the Slovenian Research Agency programme group “Medicinal Chemistry: Drug Design, Synthesis and Evaluation of Drugs” (programme code P1-0208).

P322
SCALING UP ANIMAL-FREE PBPK MODELING: CROSS-PLATFORM BENCHMARKING ACROSS ECCS CLASSES FOR NGRA APPLICATIONS ABSTRACT #471

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Background and Objectives Physiologically Based Pharmacokinetic (PBPK) modeling is increasingly used in Next-Generation Risk Assessment (NGRA) to support animal-free prediction of human pharmacokinetics using in vitro and in silico data. While several PBPK platforms are available, systematic cross-platform evaluations under standardized high-throughput workflows remain limited, particularly across compounds with different disposition characteristics. The predictive performance of automated PBPK workflows implemented in PK-Sim, GastroPlus and HTTK across Extended Clearance Classification System (ECCS) classes, was compared. Material and Methods Two publicly available pharmacokinetic datasets including Punt et al. (~42 compounds) and Terasaka et al. (~119 compounds) were evaluated. Compound-specific physicochemical and pharmacokinetic parameters from in vitro and in silico sources were harmonized across all platforms to ensure comparable parameterization. Batch PBPK simulations results were evaluated against clinical pharmacokinetic data. Predictive performance was quantified using the Geometric Mean Fold Error (GMFE). Compounds were additionally stratified according to ECCS classes to investigate differences in predictive performance according to compound disposition characteristics. Results Consistent ranking trends were observed across both datasets. For the Punt dataset, GMFE values were 3.53 for HTTK, 3.84 for GastroPlus and 4.77 for PK-Sim. Similar results were obtained for the Terasaka dataset, with GMFE values of 3.35, 4.06 and 5.03 for HTTK, GastroPlus and PK-Sim, respectively. Overall, HTTK demonstrated the most robust predictive performance under standardized high-throughput conditions, whereas PK-Sim showed larger prediction variability. ECCS-based analyses further revealed predictive accuracy differences between compound classes. Discussion and Conclusion This work presents a reproducible cross-platform benchmarking workflow for animal-free HT-PBPK modeling. The results highlight the influence of compound disposition characteristics on PBPK predictive performance and support the development of harmonized benchmarking strategies for future NGRA applications. Notably, HTTK, a free and open-source platform, demonstrated competitive predictive performance, making it a particularly attractive option for NGRA applications where accessibility and reproducibility are key requirements.

P323
AI–Based Projection of large Integrated Brain Organoid Single-Cell Datasets onto Developing Human Brain Atlas for Cell-Type Representation and Comparison of Protocol Paradigms ABSTRACT #482

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Human neural organoids derived from pluripotent stem cells have emerged as powerful in vitro systems for modeling human brain development and investigating the effects of environmental toxicants on neurodevelopment and function. Unlike traditional two-dimensional cultures, three-dimensional organoid systems capture complex cellular interactions and developmental programs that more closely resemble primary human brain tissue. These

systems are capable of modeling diverse brain regions, including cortical, forebrain, midbrain, cerebellar, thalamic, pituitary, and choroid plexus identities. More recently, increasingly sophisticated protocols have been developed to better recapitulate in vivo tissue complexity through strategies such as vascular engineering, incorporation of multiple cellular lineages, fusion of region-specific organoids, and transplantation into animal hosts. Despite these advances, quantitatively assessing the fidelity and variability of cell populations generated across organoid protocols remains challenging. Current approaches primarily rely on single-cell RNA sequencing (scRNA-seq) followed by manual or reference-based annotation of cell identities. However, these methods are limited by ambiguity in canonical marker gene expression, overlap between developmental cell states, and incomplete characterization of rare or poorly defined populations. To address these limitations, we created an integrated atlas of previously published human neural organoid scRNA-seq datasets representing more than 100 organoid differentiation protocols and totaling over 4 million cells. We applied machine learning approaches to map the transcriptomic profiles of cells from this integrated dataset onto a publicly available developing human brain scRNA-seq atlas. This framework enables identification of human developing brain cell types represented in organoids and quantitative comparison of transcriptomic similarity between primary brain and its organoid-derived counterparts across diverse developmental paradigms, including vascularized, multilineage, and assembloid-based systems. To our knowledge, this represents the largest integrated single-cell atlas of human brain organoids generated to date.

P324
ToxTransformer in NAM workflows: hazard verdicts on the Difficult Chemicals Library and mycotoxin mutagenicity transfer learning
ABSTRACT #486

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Next-generation risk assessment requires computational new approach methodologies (NAMs) that are both predictive and auditable across diverse chemical safety problems. We present two ToxTransformer use cases illustrating complementary strategies for interpretable in silico toxicology. It is a transformer-based deep learning model trained on both chemical structure and properties, output activity across 6,647 high-throughput screening endpoints. Case 1: Hazard verdicts on the Difficult Chemicals Library. The Suess et al. (2026) Difficult Chemicals Library comprises 50 deliberately challenging compounds across three types: metabolism-dependent, transport-mediated, and rare-target. We use a modular metabolism–hazard workflow that decouples bioactivation from toxicity prediction: BioTransformer enumerated Phase I/II metabolites, ToxTransformer rescored parents and metabolites against the endpoint toxicology catalog, and false-discovery-rate enrichment and specialist tools supported evidence integration. This workflow recovered metabolite-driven hazards for pro-toxicants, including methoxychlor after O-demethylation and chlorpyrifos after oxidative desulfuration, while also identifying hard failure modes: transporter-mediated organ delivery, multi-step bioactivation not generated by rule-based metabolism, and rare targets absent from the assay catalog. Case 2: Mycotoxin mutagenicity transfer learning. For 301 mycotoxins with Ames-mutagenicity labels (92 positive, 209 negative), we used ToxTransformer's outputs (top 300 biologically-relevant features, PCA-reduced) and trained student models with 10-fold stratified cross-validation. A clustering-distillation variant (PCA-guided K-means + cluster-specific feature selection) achieved AUC = 0.926 with no overfitting. Top features were dominated by yeast DNA-repair-deficiency assays (MLH1/RAD18), p53 activation, and NCI-60 cancer cell-line responses, biologically coherent with the mutagenicity mechanism. Conclusions. ToxTransformer is useful both as a direct hazard-verdict engine and as a learned featurization for downstream transfer learning. These use cases demonstrate that computational NAMs can support chemical prioritization, mechanistic hypothesis generation, and targeted follow-up assay selection, provided biological coverage, metabolic activation, chemical-domain structure, and validation design are explicitly addressed.

ECHA, EMA and EFSA perspectives on the use of NAMs

P064
EFSA's proposed assessment strategy for waiving of dog studies in pesticide approval
ABSTRACT #244

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Short-term dog oral toxicity studies (e.g., 90-day) are currently legally required for the approval process of pesticide active substances in the European Union. A retrospective analysis (1) of 473 active substances conducted by the European Food Safety Authority (EFSA) indicated that dogs did not significantly contribute to the derivation of human health-based guidance values. In line with the 3Rs principles, EFSA has proposed a New Approach Methodology (NAM)-based tiered assessment strategy (2) to support a broader paradigm shift in toxicological risk assessment, moving away from the reliance on in vivo animal studies toward less animal-intensive methodologies. In particular, the strategy includes in vitro and in silico systems to enhance the human relevance of toxicological assessments while reducing animal use. The strategy also aims to strengthen the scientific basis for waiving short-term oral toxicity testing in non-rodent (90-day dog study) species for the approval of pesticide active substances. A minimum set of data requirements, including physicochemical properties, in vitro/in silico parameters for Absorption, Distribution, Metabolism, Excretion (ADME) processes, and related Physiologically-Based Kinetic (PBK) modelling, was defined to support the approach of the strategy. A decision tree was then developed followed by testing of the strategy's feasibility and utility through proof-of-concept case studies. The proof-of-concept case study exercise demonstrated that the strategy can be effectively used and scientific justification for waiving dog toxicity studies can be provided for majority of the case studies. On the other hand, challenges and caveats of using this strategy were also identified during this exercise. Overall, this approach aligns with EU (e.g., EC Roadmap Towards Phasing Out Animal Testing for Chemical Safety Assessments) (3) and international efforts to reduce animal testing highlights the importance of advancing from animal-based toward in vitro and in silico–based risk assessment.

P065
Integration of new approach methodologies in the toxicological and allergenicity assessment of novel foods: EFSA's tiered approach
ABSTRACT #330

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The European Food Safety Authority (EFSA) has recently updated its Scientific Guidance on Novel Foods (NFs) to reflect technological and scientific advances. The revised Guidance acknowledges emerging methodologies, including new approach methodologies (NAMs), and provides insights on their consideration in the toxicological and allergenicity assessment of NFs, to support the reduction of animal testing in line with the 3R principles. EFSA recommends a tiered approach for absorption, distribution, metabolism and excretion (ADME), toxicological assessment and allergenicity. The basis for consolidating the body of evidence begins with comprehensive appraisal of existing data on the NF, its source, constituents, and potential metabolites. A read-across approach based on structurally similar compounds may also be applied. Depending on the body of evidence, in vitro methods using human cell-based systems may be considered to evaluate ADME and genotoxic potential. Where metabolite profiles differ considerably between human in vitro systems and animals,

the implications for the design and interpretation of higher-tier in vivo studies needs to be considered. Since gut microbiota affects the biotransformation of NFs and their components, and the activation or detoxification of associated metabolites, in vitro testing using models simulating the dynamic interaction of human gut and its microbiota are included in the first-tier ADME evaluation. For toxicological assessment, higher-tier in vivo studies may be triggered by first-tier findings, following a stepwise strategy. In silico, in vitro, and human studies may be required in a stepwise manner depending on the build-up evidence on allergenic potential collected at lower tiers testing, and the nature of a NF. Although NAMs are not yet fully validated for regulatory application, the updated EFSA NF Guidance foresees their gradual integration into regulatory science, as validation progresses, helping to fill gaps in risk assessment. This approach reinforces science-based decision-making while promoting the 3R principles in animal testing.

P066
Integrating new approach methodologies in the risk assessment of decontaminating substances for foods of animal origin
ABSTRACT #427

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The EFSA Guidance on the submission of data for the evaluation of the safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin intended for human consumption outlines harmonised data requirements covering technical characterisation, consumer exposure, toxicological and environmental safety, efficacy against target pathogens, and the potential for inducing reduced susceptibility to biocides or resistance to therapeutic antimicrobials. A key element of the guidance is the promotion of a tiered toxicological assessment strategy that integrates New Approach Methodologies (NAMs), including non-testing methods and in vitro assays, as starting components of hazard identification and characterisation. In line with the principles of the 3Rs (replacement, reduction and refinement of animal testing), the guidance encourages the use of non-animal approaches wherever scientifically justified, at first instance. In vitro assays, together with computational tools such as (Q)SAR models, read-across approaches and the Threshold of Toxicological Concern (TTC) principle, are highlighted as essential tools for screening genotoxicity, supporting toxicokinetic evaluation, and assessing reaction products and impurities when exposure is low. Overall, the guidance reflects the need for an integrated, science-based risk assessment approach that combines traditional toxicological testing with advanced in vitro and non-testing methodologies, ensuring robust consumer protection while supporting innovation and ethical testing strategies in food safety assessment.

Funding the development, validation and implementation of NAMs

P067
What have we achieved? A retrospective assessment of 3Rs projects funded by the set foundation
ABSTRACT #146

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Background: The Foundation for the Promotion of Alternatives to Animal Testing (set Foundation), founded in 1986 as a joint initiative of the German Federal Ministry for Food, Agriculture and Forestry and German industry associations, brings together industry, animal welfare organizations, research bodies, and government to advance the refinement, reduction, and replacement of animal experiments through the development and deployment of 3Rs methods. Objective: To assess outcomes and benefits for the 3Rs, we conducted a retrospective analysis of a

selection of recently funded projects. Methods: We reviewed project documentation and reports and surveyed project leads. Projects were categorized by project type; method developed or applied; target organ or product; field of application; and outcome. Results: The analysis shows how set Foundation support enabled promising 3Rs projects to progress into usable methods, catalyzed follow up projects, and, in some cases, contributed to OECD Test Guidelines. Notably, set funding frequently served as niche, catalytic support that positioned projects for subsequent larger grants such as from the German Federal Ministry of Education and Research (BMBF). Discussion and Conclusion: We summarize key learnings for 3Rs funding bodies, including factors that facilitate translation and regulatory uptake, and provide an outlook on the set Foundation's future plans to further accelerate impact. Keywords: 3Rs; alternatives to animal testing; impact assessment; in vitro methods; niche funding, regulatory acceptance; funding strategies; OECD Test Guidelines.

P068
Human residual tissue becomes a valuable source in scientific research ABSTRACT #161

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In The Netherlands, traditionally animal models are used in biomedical research on the etiology and treatment of diseases and in toxicological studies on constituents of food and household products. There is an urgent need to replace laboratory animals in scientific research by new approach methodologies (NAMs), preferably developed from fresh human tissue to obtain results that are more predictable for the outcome in humans and to accelerate drug development. However for scientists the access to vital human tissue is limited and needs to be improved. Fresh human residual tissue from surgery is an excellent source material for the development of various NAMs and can be obtained from virtually every organ or body part. In 2024, ETB-BISLIFE - a tissue establishment for human donor tissues - started the infrastructure VitalTissue with help of a ZonMw start-up grant. This grant was financially supported by the Dutch government and Stichting Proefdiervrij. The purpose of VitalTissue is to provide human vital residual tissue from hospitals to biomedical scientists. Earlier, a feasibility study performed by a consortium of hospitals, research- and educational institutions, companies and interest groups, had already shown that the VitalTissue initiative is organisationally, ethically and financially feasible. VitalTissue operates not for profit via a network of collaborating hospitals and research institutes. Deliveries to TNO and Utrecht University of Applied Sciences have demonstrated that residual human intestinal tissue can be successfully applied in physiologically relevant tissue models and for the development of NAMs. VitalTissue enables researchers in the public and private domains to investigate human biological processes more effectively and develop innovative NAMs. This improves and accelerates the development of new medicines and test systems, enhances the translation to human biology and facilitates the transition to animal free innovations.

P069
Landscape of challenges for in vitro testing of complex mixtures. ABSTRACT #298

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Current risk assessment guidelines primarily focus on single chemicals. Assessing complex mixtures is challenging. According to EFSA, an integrated and harmonized methodology is needed to characterize mixture components, both qualitatively and quantitatively, with particular attention to the potential presence of genotoxic and/or carcinogenic substances. Despite these efforts, a growing need remains to address existing gaps in accurately capturing the hazards of complex mixtures, particularly their combined effects. New approach methodologies (NAMs) offer promising solutions for evaluating broad toxicological endpoints without animal testing. Grouping

strategies based on shared modes of action (MOA) or adverse outcome pathways (AOP) are used to assess hazards in single molecules; however, their feasibility for mixture assessment needs to be assessed. Despite scientific progress and advocacy in the field, the regulatory use of NAMs remains limited. Beyond the application of NAMs, the limitations encountered with the application of bioassays for mixtures are also attributed to the lack of harmonized protocols for sample preparation, standardization, and validation of methods for mixtures, and data interpretation. EU-funded projects, including PANORAMIX, PARC, EUROMIX, SOLUTIONS, and MIGRATOX have advanced the application of in vitro testing for the evaluation of mixtures from “real-life” exposure scenario. These projects have supported the need of evaluating individual component interactions, synergistic and cumulative effects, and comprehensive hazard profiles, particularly for mixtures containing known or suspected toxic substances. The initiative and objectives undertaken by the Association of Official Analytical Chemists (AOAC) to address the topic of “Best Practices for Bioassay Testing of Complex Mixtures”, including challenges and opportunities, will be presented. This activity aims to raise awareness about the accurate use of bioassays for mixtures and the need for harmonization and integration of guidelines to enhance the ability to address the complexity of mixtures, identify potential hazards, and contribute to ensuring protection of human health and the environment.

P070
Comparative RNA-seq profiling of 2D, static 3D and dynamic 3D cultures and their concordance with human tissue expression
ABSTRACT #403

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Introduction Two-dimensional (2D) cell cultures continue to be widely used in cancer research and toxicology, despite their limited ability to recapitulate the structural organization and microenvironmental complexity of human tissues. Three-dimensional (3D) culture systems, including static and dynamic configurations, have emerged as more physiologically relevant platforms by better reproducing cell architecture, cell–cell interactions, and biochemical gradients. Within the CONVERT2GREEN project, preliminary transcriptomic analyses in the A549 lung cell line demonstrated that culture dimensionality and dynamic conditions substantially influence gene expression patterns. Nevertheless, a deeper transcriptomic evaluation and systematic comparison with human tissue data are essential to assess the biological relevance of these in vitro models. Objectives This study aims to comparatively characterize genome-wide gene expression profiles in 2D, static 3D, and dynamic 3D cultures, focusing on lung and liver cellular models. Furthermore, the study evaluates the concordance between in vitro transcriptomic signatures and corresponding human tissue datasets. Methods Cells were cultured under conventional 2D conditions, static 3D spheroid formation, and dynamic 3D environments using bioreactor-based systems. RNA-seq was performed to obtain global gene expression profiles. Differential gene expression, clustering, and pathway enrichment analyses were conducted using established bioinformatics workflows. Transcriptomic datasets derived from each culture configuration were compared with human tissue profiles retrieved from publicly available repositories (TCGA, SRA). Functional interpretation incorporated gene ontology enrichment and integration with curated resources, including The Human Protein Atlas and UniProt. Conclusions Comparative transcriptomic profiling provides a robust framework to evaluate how culture dimensionality and dynamic conditions shape cellular molecular phenotypes. Assessing concordance with human tissue expression signatures enables an objective evaluation of physiological fidelity, supporting the biological validation of advanced 3D and dynamic 3D culture systems. This approach contributes to the development of more predictive and

human-relevant in vitro platforms aligned with new approach methodologies (NAMs) in cancer research, toxicology and drug discovery.

P325
Toxicometabolomics as a new approach methodology for human health risk assessment of pesticides
ABSTRACT #449

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Toxicological studies based on in vitro assays and vertebrate testing face methodological limitations, including resource-intensive, costly workflows, and cross-species variability, which lacks translational capacity to identify pesticide-related health risks. Toxicometabolomics—the analysis of the metabolome to provide new sets of biomarkers for susceptibility, exposure, and effects—is gaining momentum as a New Approach Methodology (NAM) for measuring dose-response relationships and points of departure, enabling predictive insights into health hazards and risks. This systematic review evaluates metabolomics-based NAMs as potential approaches to complement, support, or safely replace animal tests required for pesticide authorization under Regulation (EU) No. 283/2013 (Article 5) and OECD guidelines. The results suggest that toxicometabolomics, in combination with other omic-based NAMs, is a suitable approach to support and, potentially, replace the existing toxicity evaluation process. Additionally, it can be used for chemical grouping (read-across). Nevertheless, adoption remains limited as the translation of metabolomics to regulatory toxicology is constrained by insufficient good practice guidelines, robust trial designs, and specialized expertise. Moreover, although untargeted metabolomics generates extensive data on the atlas of metabolic alterations, validation is needed to define relevant endpoints and ensure reliability of biomarkers for early effect detection. Additional sampling over time may be necessary to identify non-persistent biomarkers, since current studies rely on single-time-point data on the biological outcome resulting from environmental exposure.

P326
From silence to suspects: investigating cellular dialogue disruption via gap junctions in the detection of non-genotoxic carcinogens
ABSTRACT #456

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Cancer is a leading cause of mortality globally, with 20 million new cases diagnosed in 2022. This highlights the need for improved cancer prevention strategies, including the evaluation and regulation of chemicals suspected to contribute to cancer risk. Nevertheless, the identification of non-genotoxic carcinogens (NGTxCs) – substances that promote carcinogenesis without directly damaging DNA – remains a major challenge due to the absence of validated, mechanism-based non-animal testing tools capable of detecting their diverse modes of actions. One key mechanism associated with non-genotoxic carcinogenesis is the disruption of gap junctional intercellular communication (GJIC), a critical cellular process for maintaining tissue homeostasis. To date, we have investigated the effects of over 20 NGTxCs (e.g., cadmium, DEHP, TCDD, PFOA) and more than 15 non-carcinogens (e.g., D-mannitol, ampicillin, caprolactam, caffeine) on rat liver epithelial WB-F344 cells, which predominantly express connexin 43. The multiparametric Scrape Loading-Dye Transfer (mSLDT) assay has enabled the simultaneous assessment of GJIC functionality, cell density, and viability in a high-content imaging-compatible microplate format. To capture both rapid and delayed responses, cells were exposed for 30 min and 24 h, allowing

evaluation of transient as well as sustained effects. The assay currently achieves a sensitivity ~75% and a specificity >80%. Moreover, we examined cancer-relevant mechanisms from selected GJIC-inhibiting NGTxCs with distinct modes of action – including oxidative stress, inflammation, nuclear receptor signaling, cell proliferation, migration, and epithelial-to-mesenchymal transition – in both WB-F344 and ras-transformed WB-F344 cells (WB-ras). Our findings underscore the relevance of GJIC evaluation for providing mechanistic insights into carcinogenic hazard assessment. To conclude, our results support the integration of GJIC-based assays into new approach methodologies (NAMs) for NGTxCs identification and their further implementation within modular testing strategies. Supported by Czech Science Foundation Grant 24-12116S and Horizon Europe Grant 101057014 (PARC).

In vitro and in silico methods for safety assessment of medical devices

P071
In vitro sensitization assessment of medical device extracts: prevalidation results of GARDskin medical device for potential inclusion in ISO 10993-10
ABSTRACT #269

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Biocompatibility testing is essential to ensure the safety of medical devices (MDs) and skin sensitization is a key toxicological endpoint that must be assessed for all devices. For testing of neat chemicals, significant progress has been made with the development and regulatory integration of New Approach Methods (NAMs) into OECD Test Guidelines (TGs) 442C-E. These assays replace animal testing in several industry sectors. However, the regulatory acceptance of NAMs within the MD sector has progressed slowly, and testing still predominantly relies on animal-based methods, such as the Guinea Pig Maximization Test (GPMT). This study describes adaptation and prevalidation of the OECD-validated GARDskin (OECD TG 442E) for testing MDs in line with ISO 10993-10. First, the GARDskin protocol was modified to include a pre-sample extraction step using polar and non-polar vehicles according to ISO 10993-12. A feasibility study and a prevalidation study was conducted to evaluate reliability and applicability. In the feasibility study, silicone (MED-2000) and TPU materials were spiked with skin sensitizers (n=4) and extracted in both polar and non-polar vehicles (n=6). The prevalidation study comprised two steps, including a reproducibility study where a negative control material (MED-2000) was extracted and spiked with sensitizers and non-sensitizers (n=16, 3 replicates), as well as the testing of an extended dataset to evaluate concordance to animal results (n=29). The feasibility study confirmed the ability of the GARDskin MD to correctly identify sensitizers extracted from solid materials. Results from the prevalidation study demonstrated 100% reproducibility (16/16) between repeated experiments, as well as 97% concordance (28/29) to animal data. The presented results demonstrate that GARDskin MD is functional, reproducible, and accurate for assessing the skin sensitization potential of medical devices. The method fulfils all criteria to advance to an inter-laboratory ring trial to further evaluate transferability and robustness prior to potential inclusion in ISO 10993-10.

P072
Use of the 3D Reconstructed Human Intestinal Model EpiIntestinal™ for MNA Genotoxicity Assessment and Intestinal Inflammation Modelling
ABSTRACT #293

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The small intestine is a crucial organ for digestion and nutrient absorption and represents a primary site of exposure to dietary components, pharmaceuticals, and other xenobiotics. Therefore, the development of reliable, physiologically relevant in vitro models is essential for the accurate assessment of

intestinal toxicity. Cell line-based models such as Caco-2 cells, derived from colorectal carcinoma, are widely used in pharmacology but do not adequately reflect key aspects of intestinal physiology. In contrast, advanced models such as EpiIntestinal™, derived from primary non-transformed human cells, more closely mimic barrier function, metabolic competence, and inflammatory and toxicological responses. In this study, we investigated the suitability of the EpiIntestinal™ model for genotoxicity assessment using the micronucleus assay (MNA). In parallel, we evaluated whether the inclusion of inflammatory mediators, such as cytokines and chemokines, could support the use of this model as a predictive in vitro tool for the study of inflammatory bowel diseases, including Crohn's disease and ulcerative colitis. The genotoxicity protocol developed for reconstructed human epidermis (Pfuhler et al., 2021) was optimised to meet the specific requirements of intestinal tissue. Key experimental parameters, including compound application, vehicle selection, and trypsinisation time and conditions, were adjusted. The optimised protocol enabled the detection of binucleated cells containing micronuclei within the EpiIntestinal™ model, confirming the feasibility of applying the MNA with only minor modifications to the original skin-based protocol. EpiIntestinal™ was further exposed to the cytokine mixture described by Markus et al. (2021) to establish a model of the inflamed small intestine. Changes in the physiological properties of the model were assessed by measuring barrier integrity using transepithelial electrical resistance (TEER) and by immunohistochemistry. A set of anti-inflammatory molecules is currently being tested to evaluate the recovery of tissue barrier integrity and the reduction of the inflammatory response.

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P073
Replace animal tests (RAT) list – medical devices
ABSTRACT #349

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Over the last 30 years there has been significant progress in the replacement of animal use in regulatory testing; non-animal methods can now replace wholly, or in part, a number of animal tests across several sectors. Yet, animal tests persist even where non-animal methods are available. In 2015, Cruelty Free International created Replace Animal Tests (RAT) list to track such tests where animals continue to be used globally and to highlight areas of animal use that are long overdue for replacement. Through the collection and analysis of statistics on animal use, it has become apparent that regulatory testing for medical devices remains a major driver in the sustained use animals. We will present our findings, based on our RAT list database - for example, in the EU we find that medical devices drives 79% of the uses of animals in tests for skin sensitisation. We will also present an overview of the current state of the art of non-animal approaches for medical device testing. Finally, we will share our analysis of the barriers which have hindered the uptake of non-animal methods and prevented the full replacement of animal tests for medical device testing. Overall, we will show that despite the progress made in other sectors, the replacement of animal tests for medical devices severely lags behind. We will make recommendations and highlight the opportunities for progress.

In vitro systems to assess respiratory toxicity

P074
A comprehensive model for predicting the toxicology of mineral fibres by connecting the fibre parameters to the key characteristics of carcinogens
ABSTRACT #119

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Today, many research groups in the world are struggling to fully understand the mechanisms leading to the carcinogenesis of hazardous mineral fibres, like asbestos, in view of devising predictive toxicology models. Our work attempts the completion of a model aimed at evaluating how, and to what extent, physical-crystal-chemical and morphological parameters of mineral fibres prompt adverse effects leading to carcinogenesis. In vitro toxicology tests on the 10 key characteristics (KC) of carcinogens adopted by the International Association for Research on Cancer (IARC) have been performed for a commercial chrysotile divided in two size-separated fractions (> and ≤ 5 um length), standard UICC crocidolite and the non-carcinogenic wollastonite. The analysis of the in vitro data allowed to assess the major fibre parameters for each mineral fibre, and the intensity of their effect, responsible for alterations of major IARC KCs (1). Our model shows that, although similar toxicological responses of major IARC KCs are observed for the two carcinogens chrysotile and crocidolite, due their different physical-chemical features the fibre parameters contributing to carcinogenicity seem to be specific for each fibre. For chrysotile, the major fibre parameters contributing to the IARC KC cellular alterations are the surface area and the dissolution rate with the related velocity of release of metals (namely iron). For crocidolite, they are fibre length, iron, ferrous ion and transition metals content, and zeta potential. Conversely, among the 10 KCs analysed in vitro, the altered pathways discriminating between the non-carcinogenic and the carcinogenic fibres were: 2.Genotoxicity, 3.Altered DNA repair, 4.Epigenetic alterations, 5.Oxidative stress, 6.Chronic inflammation, 9.Immortalization. In conclusion, we propose the use of an algorithm for a mineral fibre predictive toxicology model by combining the fibre parameters with selected discriminating KCs, possibly leading to prevention strategies as long as the nature of the fibre of the exposed patient is known.

P075
Acellular in vitro dissolution of man-made vitreous fibers in synthetic lung fluids using single fiber and USP-4 (united states pharmacopeia apparatus 4) setups
ABSTRACT #129

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Animal studies are currently the standard for assessing the biopersistence of man-made vitreous fibers (MMVF), but increasing regulatory restrictions prompt the need for alternative in vitro methods. To be accepted, in vitro investigations must demonstrate correlation of MMVF dissolution behavior with in vivo data and thus relevant in vitro test conditions need to be found [1]. In this work, we investigated stone wool fibers dissolution using a continuous flow-through system (USP-4) and a single fiber (SF) dissolution setup. Application of USP-4 relies on indirect solubility measurements of large fiber amounts and it was successful for differentiation of MMVF with different biopersistence [2]. The SF approach offers direct observation of a single fiber dissolution via optical microscopy, closely mimicking dissolution in the lungs [3]. The aim of this work was to find agreement between stone wool dissolution behavior in USP-4 and SF. The effects of fiber composition, fluid chemistry, and the presence or removal of organic binder on in vitro dissolution of stone wool fibers were investigated. Experiments were conducted at pH 4.5 and 37° C, using simplified acellular synthetic lung fluids to simulate intracellular conditions of lung macrophages. Results demonstrated that both USP 4 and SF successfully differentiated fibers with different in vivo biopersistence. However, to achieve comparable stone wool dissolution rates in both techniques, the USP-4 procedure had to be optimized by increasing fluid flow rate-to-fiber surface area ratio (F/A) and by improving fiber wetting by adding surfactant to the fluid. This optimization also allowed testing of stone wool fibers with organic binders (as products placed on the market), showing that the presence of organic binders does not affect dissolution. The results of this study will aid

further acellular in vitro test development and harmonization between the methods for demonstrating the correlation between in vivo and in vitro data.

P077
Assessing the toxicity of traffic-derived air pollution using a primary human air-liquid interface airway in vitro model
ABSTRACT #164

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Traffic-derived air pollution (TDAP) frequently exceeds the 2021 WHO air quality guideline levels and is linked to respiratory diseases through molecular mechanisms such as oxidative stress and inflammation. To determine these mechanisms without relying on animal models and inter-species extrapolation, physiologically relevant human in vitro models are promising tools. We sought to investigate the oxidative stress and inflammatory responses to TDAP in a co-culture model of the human lung. Additionally, we aimed to examine the variability arising from different exposure days and across primary human in vitro models from different donors. Therefore, primary human bronchial epithelial cultures from three donors, each combined with primary alveolar macrophages, were exposed to a continuous flow of ambient TDAP from a high-traffic street in Düsseldorf, Germany on three consecutive days. A versatile aerosol concentration enrichment system was used to increase the fine particulate matter levels from 8 to 42 µg/m3 to 54-143 µg/m3. Gene expression of four oxidative stress markers and four inflammatory cytokines was analyzed by quantitative reverse transcriptase PCR. Compared to incubator controls, even low airflow itself induced the expression of the oxidative stress marker heme oxygenase 1 and the cytokines interleukin 8 and tumor necrosis factor alpha. TDAP exposure, compared to clean air controls, upregulated interleukin 6 in one of the three co-cultures. Because TDAP exposure had minimal effects, exposure day-specific responses could not be discerned. In four of twelve genes, we observe exposure-independet donor differences. Transcriptomic analysis suggested TDAP-induced differential expression of four lung disease-related genes which, however, could not be confirmed by qRT-PCR. Higher TDAP concentrations or repeated exposures may be required to detect robust effects in this system. Our findings highlight inter-donor variability, underscoring the need for larger donor panels. Future studies should also minimize background effects from airflow to enhance model reliability for real-time TDAP exposure studies.

P078
Respiratory uptake of inhaled dinoseb and rotenone using primary bronchial epithelial cells
ABSTRACT #168

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Inhaled substances could be taken up via the respiratory tract and reach the systemic blood circulation, leading to systemic exposure. The estimations of respiratory uptake can be improved with in vitro models of the respiratory epithelium. We have used normal human primary bronchial epithelial cells (NHBEs), cultured at the air-liquid-interface (ALI), to assess the respiratory uptake of dinoseb and rotenone. Differentiated NHBEs (three donors) were exposed to dinoseb (50, 150 or 500uM) or rotenone (1, 2 or 8uM) by applying a small droplet on the apical side of the culture (quasi-ALI). Five samples of the basolateral medium were taken over a 120-minute exposure period, additionally at the start and end of the exposure period, an apical wash, cell lysate and extract of the cell culture insert membrane were taken

for chemical analysis. Uptake of dinoseb was relatively fast, with 30-50% of the applied dose in the basolateral medium after 20 minutes for the highest dose and resulting in approximately 80% uptake after 120 minutes. The uptake for lower doses was slower (20-40% after 20 minutes) and lower (50-70% after 120 minutes). Apart from the basolateral compartment, most dinoseb was detected on the apical side of the culture, only very low quantities were detected in the cells or on the insert membrane. Uptake of rotenone, measured by the fraction in the basolateral compartment, was lower than for dinoseb. Highest quantities of rotenone were retrieved in the cellular fraction and the apical wash, whereas quantities in the basolateral compartment were often below the detection limit. Our data can be used to parameterize physiologically-based kinetic models to estimate systemic concentrations of inhaled substances for toxicological assessment. As such, in vitro respiratory models not only play a role in the identification of local hazards, but can also provide systemic concentrations after different exposure scenarios.

P079
Advancing non-animal respiratory toxicology: a TRPA1-based in vitro model for airway irritant detection
ABSTRACT #175

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Environmental exposure to volatile airway irritants can elicit sensory irritation and contribute to acute respiratory outcomes such as pain, cough, dyspnea, and bronchospasm, as well as chronic conditions including asthma, airway hyper-responsiveness, and chronic obstructive pulmonary disease (COPD). The transient receptor potential ankyrin 1 (TRPA1) ion channel, expressed in airway sensory neurons, is a key molecular sensor of such irritants. Its activation, leading to calcium influx and substance P release, constitutes the molecular initiating event of Adverse Outcome Pathway 196 (AOP 196). Current approaches to assess irritancy rely on in vitro assays for skin and eye corrosion, while respiratory irritation testing remains largely restricted to the in vivo RD50 assay, which measures a 50% reduction in respiration rate. Although the RD50 correlates with human irritancy for some compounds, it lacks mechanistic resolution, is unsuitable for high throughput screening, and may underestimate risks, as suggested by epidemiological evidence of elevated pulmonary disease incidence in occupationally exposed populations despite adherence to occupational exposure limits. Here, we present the development of a human relevant in vitro model for airway irritant detection anchored in AOP 196. Using the alveolar epithelial cell line A549, which expresses functional TRPA1, we demonstrate that irritant exposure induces TRPA1 activation and downstream upregulation of the substance P gene (TAC1). The assay robustly identified prototypical irritants while showing minimal responses to non irritant controls, and results were concordant with in vivo RD50 data. This TRPA1 based in vitro assay provides a mechanistically anchored, high throughput alternative to animal based respiratory irritation tests. By leveraging the AOP framework, it offers a promising tool for chemical safety assessment and regulatory decision making in respiratory toxicology.

P080
Assessing inhalation toxicity of cosmetic compounds on upper airway and alveolar air-liquid interface models after liquid or nebulized exposure
ABSTRACT #192

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While dermal absorption remains the primary route of exposure for cosmetic products, incidental inhalation represents a relevant secondary pathway - particularly for sprayable products such as deodorants or hair styling sprays. Inhalation exposure can lead to local irritation in both the upper respiratory tract (nasal and bronchial epithelium) and the deep lung (alveolar region), depending on particle size and compound characteristics. However, standardized in vitro protocols to assess these toxic effects using New Approach Methodologies (NAMs) are still lacking. To address this gap, we employed two human-derived air-liquid interface (ALI) models - MucilAir™ (upper airways) and AlveolAir™ (alveolar region) - to evaluate three cosmetic ingredients with known in vivo inhalation toxicity

profiles. For each compound, five concentrations were selected based on estimated regional deposition derived from historical rat inhalation data. Exposure was conducted via both liquid and nebulized application to compare technical feasibility, reproducibility, and biological relevance. Toxicity was assessed using a comprehensive panel of endpoints, including transepithelial electrical resistance (TEER), lactate dehydrogenase (LDH) release, and cytokine secretion. Preliminary results show promising concordance between in vitro responses and known in vivo toxicity profiles, supporting the potential of these models to derive safe exposure concentrations. Variability in responses between the two airway models and exposure modalities will need to be systematically evaluated to support further protocol refinement. This approach represents an important step towards establishing a standardized, animal-free testing strategy for inhalation safety assessment of novel sprayable ingredients.

P081
Breathing small airways lung-on-chip to model COPD pathophysiology and assess lung toxicity
ABSTRACT #193

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Chronic Obstructive Pulmonary Disease (COPD) remains a global health challenge with no curative therapies available and cigarette smoke is recognized as one of the major risk factors (1). To develop innovative treatments, it is fundamental to advance the understanding of disease's mechanisms through advanced in vitro models, such as Organs-on-chip (OoC). Here we present a COPD-OoC model, generated by co-culturing human bronchial epithelial cells (HBECs) and smooth muscle cells (BSMCs) in a 3D microenvironment, where a physiological-like mechanical stimulation mimics the natural respiration. The BMSCs were organized as 3D microtissues, while HBECs were lined along the muscles, generating epithelial monolayers. The stromal and epithelial compartments were firstly optimized by culturing the cells for 14 days both in static and dynamic (i.e. cyclic sinusoidal 10%uniaxial strain, 16bpm) conditions. For the stromal compartment, a seeding density of 50x106cells/mL of BSMC led to high cell viability and interconnectivity, with BSMC elongating and expressing muscular markers (a-SMA, b-tubulin and Collagen I) especially after mechanical stimulation. For the epithelial compartment, a seeding density of 10x106cells/mL yielded optimal cell viability and barrier-like structure formation (ZO-1 expression). The optimized compartments were finally combined in a 5-channels device compatible with air-liquid-interface, encompassing two BMSC microtissues separated with HBECs monolayer. The model correctly developed in 10 days: BSMCs expressed a-SMA and Collagen I and exhibited increased elongation when subjected to mechanical stimulation; epithelial cells formed structured monolayer and expressed epithelial cells sub-populations markers, such as MUC5B and SCGB1A1 (acinar-cells) and FOXJ1 (ciliated-cells). The obtained mechanically active system was exposed to pro-inflammatory cytokines (IL-13) and/or to cigarette smoke extracts and their effect in inducing COPD traits or cell toxicity was assessed. By closely replicating the pathological microenvironment of COPD, this model offers a powerful tool for assessing lung toxicity and studying disease mechanisms, to finally accelerate drug discovery.

P082
In silico evaluation of inhalation exposure to essential oils
ABSTRACT #202

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Essential oils (EOs) are widely used in aromatherapy, cosmetics, perfumes, and air fresheners. They contain numerous volatile compounds, which can lead to inhalation exposure. Exposure levels depend on chemical properties, product formulation, physiological factors, and the specific exposure scenario. When direct analytical measurements are not available, simple mathematical models can provide exposure estimates. Two scenarios have been considered. The first involves topical

application, such as a roll-on on the forearms. The SCCS equation provides a rough estimation by dividing the exposure period into two phases: a “close-range” phase, with chemical diffusion near the nose, followed by a “room-scale” phase, accounting for broader diffusion. Key variables include the respirable and airborne fractions of the product. The second scenario models emission from a single source in a room, such as a diffuser. A differential equation describes chemical emission and removal via inhalation and ventilation. The model assumes instantaneous homogenization and no chemical stratification, meaning that exposure is independent of distance from the source. These models enable practical estimation of inhalation exposure without complex measurements. Equations are provided, along with calculation examples using real products, illustrating their application for both topical and diffusion EO exposures.

P083
Towards prospective identification of respiratory sensitizers: setting up a co-culture of bronchial epithelial and dendritic-like cells
ABSTRACT #210

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Respiratory sensitizers (RSs) are the primary contributors to occupational asthma. While it is important to prospectively identify RSs, there is currently no OECD-approved test guideline available for this purpose. In recent work we tested the bronchial epithelial cell line Calu-3 at the air-liquid-interface as an in vitro model for identification of RSs. We exposed the cells to three chemicals: chloramine-T, piperazine and 2,4-toluene diisocyanate. Even though they are all RSs, only piperazine showed a dose-dependent increase in IL-6 response, indicating that the other chemicals may induce RS through a different mechanism. Given that activation of dendritic cells (DCs) is acknowledged as a KE in the AOP for respiratory sensitization, in the current work we extended the Calu-3 model with THP-1, a monocytic cell line that can serve as a surrogate for DCs, to improve the identification of RSs. Similar to previous experiments mitochondrial activity (WST-1), LDH release and barrier integrity (trans-epithelial electrical resistance, TEER) was measured in the co-culture. Additionally, surface marker expression (CD54, CD86, and TSLPr) on THP-1 cells was analyzed using fluorescence-activated cell sorting (FACS), and a more comprehensive panel of 14 cytokines was measured. Preliminary measurements indicate that piperazine exposure does not affect barrier integrity, LDH release and mitochondrial activity, which is in line with the results from the Calu-3 monoculture. Additionally, FACS could not elucidate exposure effects relative to the air control. In contrast to the monoculture, where TDI induced effects only at cytotoxic dose levels, the co-culture seemed to exhibit alterations in surface marker levels already at sub-cytotoxic concentrations. Currently, experiments are ongoing to confirm these preliminary findings. While these initial findings are encouraging and suggest that the co-culture model may help identify RSs that act via DC-related mechanisms, additional data will be needed to draw definitive conclusions.

P084
The exposure to inhalable substances and their impact on the safety assessment of cosmetic products and their ingredients
ABSTRACT #219

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Background and Objectives Inhalation exposure to cosmetic ingredients can pose health risks, especially in sprays and powders where airborne particles reach the lower respiratory tract. Assessing the safety of titanium dioxide (TiO_2), quartz, and Aerosil (amorphous silica) is crucial to ensure their safe cosmetic use. This study aimed to evaluate the effects of these substances on cellular viability and epithelial barrier integrity using a human three□dimensional (3D) bronchio□alveolar tissue model (EpiAirway) at a relevant concentration of 120 ppm. Materials and Methods The EpiAirway model (MatTek, AIR□100) consists of highly differentiated human tracheobronchial epithelial cells forming multiple layers with tight junctions and beating cilia, realistically reproducing the respiratory epithelium. Tissues were treated every 48 hours with TiO_2, quartz, or Aerosil diluted in EpiAirway MUCUS (1:10 in TEER buffer). Barrier integrity was monitored through transepithelial electrical resistance (TEER) measurements on days 2, 5, and 7, while cell viability was evaluated by the MTT assay at day 7. Data were normalized to the negative control and analyzed in triplicate. Results TEER measurements showed no significant differences between treated tissues and controls at any time point, indicating preservation of epithelial barrier function. The MTT assay revealed that TiO_2 and Aerosil did not reduce cell viability, while quartz caused an average 27% decrease compared with the control, suggesting a potential cytotoxic effect. Discussion and Conclusion The results confirm the suitability of 3D tissue models as advanced tools for inhalation safety assessment of cosmetic ingredients, offering more predictive data than traditional 2D systems and aligning with the ethical principles of the 3Rs. Exposure to TiO_2 and Aerosil did not compromise tissue viability or integrity, whereas quartz induced moderate cytotoxicity that warrants further investigation into possible inflammatory, oxidative, and transcriptomic responses.

P085
Effects of ozone on lung cells exposed at the air-liquid interface under controlled conditions.
ABSTRACT #227

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Air pollution caused by particles and gases in the atmosphere is responsible for a significant number of premature deaths. In 2022, the World Health Organisation (WHO) estimated that 99% of the global population was breathing polluted air [1]. Among gaseous pollutants, tropospheric ozone has been demonstrated to exacerbate respiratory diseases, most notably asthma and chronic obstructive pulmonary disease, and could to be responsible for 70,000 premature deaths per year in Europe [2]. Comprehending the mechanisms underlying ozone toxicity represents a significant challenge. The study of the effect of ozone on bronchial and alveolar epithelial cells, cultured in an air-liquid interface (ALI), was conducted by exposing the cells using a Vitrocell® system. The cells were exposed to an ozone concentration of 100 and 120 µg/m3 for 2 hours. The parameters that were measured included epithelial barrier integrity (as indicated by paracellular permeability to Lucifer Yellow), cellular stress (as indicated by lactate and mucin levels), and inflammatory response. These measurements were taken 6 hours and 24 hours after the start of exposure. Preliminary findings indicate the activation of specific mechanisms under investigation, including the inflammatory response, and a cytotoxic effect during the first hours. Following a 24-hour period, a number of the parameters under investigation have been observed to revert to levels that approximate those of cells exposed to zero air. The issue of repeated exposure appears to be a concept of considerable interest for our preliminary work. This work is part of the ‘Territoires d'Innovation: Dunkerque, l'énergie créative’ project, funded by the ‘Grand Plan d'Investissement’ and the ‘Oz-Tox’ project, funded as part of the CPER ECRIN, with the participation of the Observatoire Local de Santé de l'Espace Santé du Littoral.

P086
Phthalates alone or in a mixture induce mitochondrial dysfunction and phenotypic modification in bronchial cells
ABSTRACT #231

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Phthalates are organic compounds used in plastic polymers to enhance flexibility, transparency, and malleability. Compounds such as bis(2-ethylhexyl) phthalate (DEHP) and diisobutyl phthalate (DiBP) are frequently detected in both outdoor and indoor environments [1]. Inhalation represents an important route of exposure for the respiratory epithelium. Several studies have shown that phthalates can impair mitochondrial function and induce oxidative stress, suggesting a key role for mitochondria in their cellular toxicity [2], [3]. However, the combined effects of these compounds remain poorly understood. This study aimed to determine the effects of phthalates, alone or in mixture, on the human bronchial epithelial cell line BEAS-2B in vitro. Different metabolic parameters were measured, including intracellular adenine nucleotides (ATP, ADP, AMP) by LC-UV, lactate and pyruvate production by LC-MS/MS, oxygen consumption rate and extracellular acidification by Seahorse analysis, as well as total and mitochondrial ROS levels and cell cycle distribution by flow cytometry. Our first results showed that phthalates—particularly mixtures dominated by DEHP—alter mitochondrial function, as evidenced by a decrease in the ATP/ADP ratio, reduced oxygen consumption, and increased extracellular acidification and lactate production. These findings indicate a metabolic shift from oxidative phosphorylation toward glycolysis. This reprogramming was accompanied by elevated ROS production in both cytoplasmic and mitochondrial compartments and by disruption of the cell cycle. In conclusion, inhaled phthalates can target mitochondria in bronchial epithelial cells, leading to metabolic reprogramming and oxidative stress, highlighting mitochondrial dysfunction as a key mechanism of phthalate-induced toxicity in airway cells. Further investigations are currently underway to explore additional mitochondrial mechanisms involved in phthalate-induced toxicity.

P087
Mixture toxicity of benzene, toluene, ethylbenzene, and xylene (BTEX) in a human organotypic model of the respiratory tract exposed acutely or repeatedly at the air/liquid interface ABSTRACT #243

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The impact of volatile organic compounds (VOCs) and, more broadly, air pollution on the respiratory system, as well as the increasing prevalence of diseases such as asthma, is a concerning issue. Benzene, toluene, ethylbenzene, and xylene (BTEX mixture) are the most representative aromatic VOCs in terms of occurrence frequency and health impact. Acute exposure to individual BTEX compounds can lead to irritation, affect the central nervous system and the respiratory system. However, very few experimental studies have examined the impact of BTEX as a mixture on in vitro models at low concentrations. This study investigates the impact of BTEX compounds on a human organotypic cellular model of the respiratory tract in order to identify the mechanisms of toxic action, and the potential cocktail effect. In particular, could the mechanisms identified explain the association found between exposure to BTEX and the onset of asthma in children? The effects of BTEX on the MucilAir™ model was determined, both through individual exposures and as a quaternary mixture at realistic concentrations, using a physiologically based pharmacokinetic (PBPK) model. Exposures were conducted at the air-liquid interface (ALI) using the Vitrocell® system, for 4 hours under acute conditions and 4 hours per day over three consecutive days for repeated exposures. Quantifying inflammatory markers via ELISA and analyzing membrane integrity, transcriptome and proteome analyses were performed. A stable and robust dynamic atmosphere generation system was developed

to enable realistic exposure of cellular models. Repeated exposure to these toxicants led to functional and structural alterations in the epithelium. Repeated exposure to BTEX induces sustained activation of the inflammatory response (IL-6, IL-8), especially with benzene and the mixture, associated with a moderate but noticeable alteration in epithelial barrier function and the ciliary function, as shown by ELISA and RNAseq. This could be related to the induction of asthma.

P088
Study of the toxicity of prenol, a second generation biofuel, by coupling an atmospheric simulation chamber to an air-liquid interface exposure device ABSTRACT #248

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Background and Objectives: Air pollution affects air quality, climate, and human health, and the use of biofuels has therefore grown considerably. One candidate for second-generation biofuel is prenol (3-methyl-2-buten-1-ol). However, this Volatile Organic Compound (VOC) could be released into the atmosphere while it is transported, stored, or unburned in automobile engines. Then, prenol reacts with atmospheric oxidants, which can lead to the formation of several gaseous oxidation products as well as Secondary Organic Aerosols (SOAs), that are potentially hazardous. The objective of this study was to assess, in vitro, the toxicologic effects of prenol and its ozonolysis products. Material and Methods: The ozone reaction of prenol was performed in the atmospheric simulation chamber CHARME (Dunkerque, France). A PTR-ToF-MS and a SMPS were used to monitor the concentrations of VOCs and SOAs, respectively. To investigate the impact of prenol and its ozonolysis products on health, an air/liquid interface (ALI) cell exposure device (Vitrocell) was coupled to CHARME to expose human lung cells (A549 cell line) for 2 hours to prenol, ozone or prenol+ozone. Results: The major oxidation products detected in the gas phase were methylglyoxal, glycolaldehyde, and acetone. The formation of SOAs was also observed. In addition, no significant cytotoxicity following any of the exposures was detected. Comparing the cells exposed to prenol, ozone or both in combination with filtered air used as a control, transcriptomic analyses revealed gene induction of proteins involved in xenobiotic metabolism and antioxidant defense. Additionally, a pro-inflammatory response was measured at the protein and gene levels. Discussion and Conclusion: This study is the first coupling of an ALI exposure device to an atmospheric simulation chamber for studying the VOC toxicity. The main pathways modified by exposure to prenol concerned either the cell cycle, differentiation, structural modification, cell death and cancer pathways, or oxidative and inflammatory diseases.

P089
In vitro immune off-target toxicity assessment using morph_ONE for antibody drug conjugates (ADCs) ABSTRACT #249

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Antibody drug conjugates (ADCs) offer targeted delivery of cytotoxic agents to cancer cells, improving therapeutic precision. However, off-target toxicities remain a major challenge, with interstitial lung disease (ILD) recognized as one of the most severe adverse events. ILD involves inflammation and fibrosis of pulmonary tissue, potentially leading to respiratory failure and fatal outcomes. Its mechanisms are not fully understood but may include immune-mediated responses, non-specific uptake, and systemic exposure to the payload. This study aimed to (i) optimize and validate a human in vitro system using alveolar macrophages to predict pulmonary toxicity observed in in vivo ADC studies, and (ii) assess the impact of ADCs versus individual components on

respiratory inflammation and macrophage morphology. ImmuPHAGE™ (human alveolar macrophage-like cells, ImmuONE, UK) were exposed to ADCs with known lung toxicity (trastuzumab deruxtecan, brentuximab vedotin, and sacituzumab govitecan) at concentrations of 0.001–100 µg/mL for up to 144 h. High-content imaging (InCell Analyser 6000) assessed cell health and morphology, while cytokine secretion was measured using MSD multiplex assays. Results showed that free drugs caused dose- and time-dependent cytotoxicity, reducing cell count and mitochondrial activity, whereas ADCs did not significantly impair cell viability. However, ADC exposure induced morphological changes, notably foamy macrophage appearance and increased vacuolation in a dose-dependent manner. Cytokine analysis revealed elevated proinflammatory markers, particularly IL-6 and TNF-α. These findings demonstrate that the morph_ONE high-content imaging assay is effective for detecting macrophage health and morphological alterations linked to ADC treatment. Incorporating this tool early in ADC development could help identify potential pulmonary risks before animal studies, improving candidate selection and enhancing drug safety.

P090
An advanced airway-on-a-chip model with unidirectional flow and perfusable microvascular network for studying fibrosis
ABSTRACT #264

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Developing physiologically relevant in vitro models of the human lung is essential for studying respiratory diseases and testing therapeutic interventions. Current models either prioritize biological complexity at the expense of scalability or adopt simplified membrane-based designs that lack dynamic multicellular interactions and functional vasculature. These limitations are particularly evident when modeling complex pathologies such as interstitial lung diseases, including pulmonary fibrosis. To address these limitations, we developed a respiratory airway model on the OrganoPlate Graft UF 32 platform that integrates an airlifted pseudo-stratified bronchial epithelium supported by a stromal layer and a fully perfusable microvascular system under unidirectional flow. This design closely mirrors the intricate organization and key features of native lung tissue. As a proof of concept, we induced fibrosis using a well-defined cocktail (FC) of compounds and comprehensively evaluated key features such as epithelial-mesenchymal transition (EMT), epithelial barrier integrity (measured by Trans-Epithelial Electrical Resistance, TEER), vasculature rearrangement, and perfusability changes. The platform seamlessly integrated biological complexity with scalability, accurately recapitulating the intricate features of the lung microenvironment while remaining compatible with automated high-throughput systems. The model demonstrated robustness and longevity, maintaining a differentiated, airlifted bronchial epithelium supported by a stable, perfusable microvascular network for over 30 days in culture. Exposure to the FC led to significant changes, including alterations in EMT marker expression and barrier function, modified cellular architecture, and the induction of inflammatory markers. Additionally, the model's perfusable microvascular network enabled the successful perfusion of immune cells, adding an important dimension for studying immune cell dynamics and interactions within the lung microenvironment. This in vitro model represents a crucial advancement by incorporating unidirectional flow and replicate the spatial complexity of the respiratory airway through the integration of relevant cell types in close proximity, supported by a functional microvascular system.

P091
A novel adverse outcome pathway for respiratory sensitisation: challenges and opportunities for in vitro safety assessment using new approach methodologies (NAMs)
ABSTRACT #265

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Chemicals that become bioavailable through respiratory exposure can trigger immunological responses, leading to irritation or sensitization. Severe reactions may result in pronounced and potentially life-threatening hypersensitivity conditions, particularly when exposure exceeds occupational exposure limit values. Repeated exposure in occupational settings can lead to chronic respiratory diseases, posing significant public health risks (e.g., diisocyanate-induced asthma, lung inflammation, occupational bronchitis, chronic obstructive pulmonary disease, pneumoconiosis, hypersensitivity pneumonitis, etc.). Occupational lung diseases can be triggered by exposure to a wide range of substances and conditions, for example in the construction industry, as well as in mining, agriculture, and other professions. Many chemicals, including those present in consumer products, have never been assessed for respiratory sensitization despite their potential hazard. Current testing approaches, particularly in vivo animal testing, are increasingly considered outdated and of limited predictivity for human health risk assessment. We propose the use of advanced technologies (New Approach Methodologies, NAMs), such as three-dimensional respiratory epithelial tissue models that secrete relevant biomarkers (e.g., cytokines and chemokines), to establish a more human-relevant in vitro system. Our pilot data indicate that combinations of these technologies show promise in predicting respiratory sensitization at an early stage, offering a more accurate and ethical alternative to animal testing. The primary aim of our work is to contribute to addressing critical gaps in current knowledge in the safety assessment of chemicals with the potential to induce allergic immune responses. Through the use of NAMs, we aim to support the regulatory acceptance of these advanced testing strategies, leading to long-term improvements in global public health protection. This work was supported by the Ministry of Health of the Czech Republic – DRO ("National Institute of Public Health – NIPH, IN: 75010330").

P092
Establishment of a human pulmonary in vitro co-culture model for nanoplastic toxicological assessment: calu-3 and THP-1 macrophages ABSTRACT #267

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Plastic can be degraded with weathering into micro and nanoplastics (MNPLs) and be spread worldwide, ultimately reaching living organisms through air, water, and food sources. Apart from ingestion and dermal absorption, inhalation is one of the main routes of human exposure to MNPLs. Despite its particular concern, limited information has been collected regarding the interaction of MNPLs with the lung epithelium, leaving critical gaps in our understanding of their potential effects on respiratory health. Therefore, there is an urgent need to develop new approach methodologies (NAMs) capable of better mimicking human epithelia in vitro as a model to test MNPLs. Calu-3 is a lung adenocarcinoma cell line that can be cultured under air-liquid interface (ALI) conditions, exhibiting respiratory tract features that remain stable for extended periods. However, airway epithelial cells do not function in isolation, the immune system is also present in the lungs. To enhance the reliability of this model assessing MNPLs pulmonary effects, a co-culture of Calu-3 cells with THP-1 macrophages are established under ALI conditions. To test the new model robustness several endpoints were assessed, including the capability of generating stable tight junctions, barrier permeability, microvilli formation, macrophage-epithelial cell interaction and cytokine release. Following the standardization of the model, it was used to assess the effects of MNPLs on the barrier after two weeks of repeated exposures. Preliminary results demonstrated the impact of polyethylene terephthalate nanoplastics (PET-NPLs) on the barrier, highlighting the coculture model as a reliable NAM for testing the effects of repeated MNPL exposures on the lung epithelium.

P093
Evaluation of Novel SARS-CoV-2 Mpro Inhibitors Using Computational and In

Vitro Approaches
ABSTRACT #296

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The safety and efficacy of drugs, including antivirals, are cornerstones of modern pharmacy and medicine. The outbreak of the COVID-19 pandemic in 2020 highlighted the urgent need for an accelerated screening process for novel drug candidates. New approach methodologies and advanced computational methods were applied to identify the most promising molecules. In this context, we investigated a series of newly synthesised benzoazepine derivatives for their ability to inhibit Mpro, the SARS-CoV-2 main protease as an antiviral target. At the same time, we evaluated their potential adverse effects in target organs, taking into account the hypothesised routes of administration, namely inhalation and oral intake. In silico modelling was performed using docking simulations (Viskupicova et al., 2023) to examine interactions within the catalytic site of Mpro. The most promising candidates were subsequently subjected to an enzymatic inhibition assay, followed by in vitro toxicity screening. In silico modelling was performed using docking simulations (Viskupicova et al., 2023) to examine interactions within the catalytic site of Mpro. The most promising candidates were subsequently subjected to an enzymatic inhibition assay, followed by in vitro toxicity screening. Cytotoxicity was assessed in VeroE6 cells using the Neutral Red Uptake assay, enabling the determination of IC50 values and the selection of testing concentrations for more complex in vitro 3D models of the lung (EpiAirway) and small intestine (EpiIntestinal). Inhalation exposure was performed using the VITROCELL® Cloud MAX aerosol exposure system. Exposure levels were defined based on IC50-guided dosing, using 1×, 5×, and 10× concentrations established in the VeroE6 cell line. Across all tested conditions, none of the compounds reduced tissue viability below 80%, even at the highest concentrations applied. Furthermore, no significant increases in the production of pro-inflammatory cytokines (IL-1α and IL-8) were detected, suggesting a low irritation potential. Although the observed inhibitory activity against Mpro was moderate, the favourable safety profile of these compounds supports their further optimisation to enhance antiviral potency. These results demonstrate the utility of integrating computational approaches with human-relevant in vitro models for the efficient identification and prioritisation of safe antiviral candidates, while supporting the development of more predictive and streamlined drug discovery pipelines.

P094
Cooling agents in e-cigarettes: toxicological evaluation of WS-3, WS-5 and WS-23 in bronchial epithelial models
ABSTRACT #310

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E-cigarette use is increasing in Germany. Between the years 2023 and 2024 the prevalence of e-cigarette users among the adult population was estimated to be one million. They are most prevalent among smokers due to the colorful packaging and various flavors, such as fruit, dessert, menthol, and mint. E-cigarettes may contain cooling agents such as WS-3, WS-5, and WS-23, as well as menthol. Cooling agents activate TRPM8 ion channels on sensory nerve endings responsible for cold perception. This gives e-cigarette users a cooling sensation without actually decreasing the temperature. However, most toxicological studies of cooling agents focus on oral consumption. There is a significant lack of data on inhalation effects, making it difficult to determine the safety of these substances. This study aims to evaluate the toxicity of cooling agents commonly found in e-cigarette liquids and assess their potential risk to human health. For this study, bronchial epithelial cells BEAS-2B were cultured and exposed to various concentrations and compositions of laboratory-made e-cigarette liquids. Some liquids contained nicotine, while others did not. The cells were tested for cytotoxicity using the PrestoBlue assay, and inflammatory processes were analyzed by measuring cytokine concentrations. The LEGENDplex HU Essential Immune Response

Panel was used for quantitative measurements of multiple cytokins/chemokens (IL-4, IL-2, IP-10, IL-1b, TNF-α, MCP-1, IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, IL-8, Free active TGF-β1) in samples via fluorescence-activated cell sorting (FACS). The results showed that the cooling agents WS-3, WS-5, and WS-23 reduced the metabolic activity of cells depending on the concentration. Nicotine enhanced the negative effect of cooling agents on cell metabolic activity, and pronounced cytokine secretion was observed in all cases. Under these conditions, MCP-1 was almost completely suppressed while IL-6 and IL-8 levels either remained elevated or increased further compared to propylene glycol/vegetable glycerin (PG/VG), which was used as a control.

P095
Transcriptomic and cytokine responses of reconstructed human bronchial epithelium to respiratory versus skin sensitizers
ABSTRACT #315

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Background and Objective: Exposure to respiratory sensitizers is associated with significant public health concerns, including asthma and rhinitis. While skin sensitization tests are known to yield positive responses to respiratory sensitizers, no validated in vitro tests currently exist to distinguish respiratory sensitizers from skin sensitizers. This study aims to develop an in vitro respiratory sensitization test capable of distinguishing respiratory sensitizers from skin sensitizers using reconstructed human bronchial epithelium (RhBE). Materials and Methods: Primary human bronchial epithelial cells were cultured at the air–liquid interface for 28 days to generate mucociliary-differentiated RhBE. Two skin sensitizers (1-chloro-2,4-dinitrobenzene and ethylene glycol dimethacrylate) and four respiratory sensitizers (chloramine T, piperazine, trimellitic anhydride, and hexamethylene diisocyanate) were applied to the differentiated RhBE. Four hours after exposure, cell viability was analyzed using the MTT assay. RNA□sequencing analysis was performed at test chemicals concentrations that maintained >80% cell viability. Cytokine concentrations in culture supernatants were analyzed using ELISA. Results: In the MTT assay, all test chemicals reduced cell viability to <50% at the highest concentrations. RNA□sequencing analysis revealed that two heat shock genes (HSPA2 and HSPA8) were commonly up-regulated following exposure to all sensitizers. Several genes including ARG2, MALAT1, and MMP1 were specifically up-regulated by all four respiratory sensitizers. ELISA results showed that IL-8 concentrations increased in response to all sensitizers, whereas IL-33 was markedly increased only following exposure to piperazine. Discussion and Conclusion: RhBE exhibited distinct transcriptional and cytokine responses to respiratory sensitizers compared with skin sensitizers. These differences may serve as candidate biomarkers for distinguishing respiratory sensitizers from skin sensitizers. Various groups are currently developing in vitro methods using RhBE to assess inhalation toxicity. Our findings suggest that RhBE could be a useful platform for evaluating immunological toxicity associated with inhalation exposure to chemicals.

P096
The USP-4 as a tool to assess the dissolution of man-made vitreous fibres
ABSTRACT #332

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Man-Made Vitreous Fibres (MMVF), including glass, stone, and slag wool fibres, are extensively utilized as insulation materials in the construction sector. During their production and handling, a small portion of MMVF can become airborne, posing a risk of inhalation by humans. When fibres are inhaled, their durability within the lung environment plays a crucial role in long-term health outcomes, according to the fibre paradigm. This insight guided the International Agency for Research on Cancer (IARC) to classify low-biopersistence MMVF as Group 3 in their 2002 evaluation [1]. Currently, biopersistence assessments largely depend on in vivo testing. Although in vitro methods for evaluating biodurability have been widely used by researchers and industry to study fibre persistence in the lungs, these techniques have not yet gained regulatory acceptance as substitutes for in vivo tests. A significant limitation of existing methods is their inconsistent reproducibility both within and between laboratories [2]. The European Insulation Manufacturers Association (EURIMA) is working on developing an in vitro test to measure MMVF dissolution [3]. The USP Apparatus 4 (USP-4), a device recognized by both the US and European Pharmacopoeia for standardized medicine dissolution testing, is being employed in this effort. This study examined commonly used simplified lung fluids for in vitro acellular testing of glass and stone wool fibre dissolution at pH 7.4 (extracellular) and pH 4.5 (intracellular) conditions. The evaluation considered the fluids' chemical makeup, pH buffering capacity, and their ability to distinguish between the dissolution rates of low- and high-solubility fibres. From this study, a shortlist of candidate fluids was identified for protocol optimization in future work, focusing primarily on choosing between closed or open loop configurations.

P097
Estimation of respiratory irritation potency of industrial chemicals by using the TRPV1 expressing SH-SY5Y cell model and machine learning
ABSTRACT #342

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Chemosensory irritation in the airways is commonly measured in mice or rats that are exposed to compounds via inhalation, and the concentration generating 50% respiratory depression (RD50) is determined. As an alternative to the RD50 test, the neuronal SH-SY5Y cell model expressing the nociceptor TRPV1 was used to study 34 organic compounds with a wide range of RD50 values (1). Upon acute exposure to chemicals, the Ca2+ influx was monitored in the TRPV1-SH-SY5Y cells, without and with the TRPV1 antagonist capsazepine (CZ). The concentration-responses were normalized to the response induced by the TRPV1 agonist capsaicin and the EC20, Emax and the concentration generating Emax were estimated without and with CZ. These values, together with scores from principal component analyses, were then used for prediction of chemosensory irritation potency, using random forest machine learning analysis. Three data sets were used to develop the models; all data, data without (no CZ) or data with CZ (+CZ). Based on RD50 values from literature, a 2-class model predicting severe irritancy and intermediate/non-irritancy and a 3-class model predicting severe, intermediate and non-irritancy were developed. The best 2-class prediction model was the one without TRPV1 influence (90% accuracy for +CZ, vs. 87% for all data and 81% for no CZ), whereas TRPV1-mediated Ca2+ influx (no CZ) improved the accuracy from 71% to 77% in the 3-class model. In the 3-class model, 4 chemicals were

classified as more irritating than in the RD50 test in vivo. Three severely irritating chemicals were erroneously classified as non-irritating by the cell assay. In conclusion, the cell model was able to classify respiratory irritation with high accuracy, but severe irritation was not mediated by TRPV1 activation. A few chemicals induce respiratory irritation by a mechanism that cannot be determined in the cell model, e.g. by TRPA1 activation.

P098
Single-cell mass cytometry reveals multicellular context–dependent reshaping of oxidative stress–related key events following Ceria nanoparticle exposure
ABSTRACT #372

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Ceria nanoparticles (CeNPs) are redox-active nanomaterials with reported antioxidant properties; however, pulmonary toxicity studies have produced contradictory outcomes, ranging from cytoprotection to enhanced oxidative damage. These inconsistencies suggest that CeNP effects are highly context-dependent, and that cellular microenvironment may critically modulate oxidative stress–driven adverse outcome pathways (AOPs) beyond particle properties alone. Despite this, nanotoxicity assessment still predominantly relies on mono-culture systems and bulk endpoints, which fail to capture alveolar multicellular complexity, intercellular communication, and cell-state–dependent stress responses, limiting their predictive value as new approach methodologies (NAMs). In this study, we compared mono-culture and a 3D alveolar tetra-culture system under identical oxidative stress and CeNP exposure conditions to determine how culture complexity reshapes oxidative stress–related key events. Human lung epithelial (A549), endothelial (EA.hy926), and immune-relevant cells were cultured individually or combined in a 3D tetra-culture system, primed with tert-butyl hydroperoxide followed by CeNP exposure (5 ppm, 3 h), and analyzed by single-cell mass cytometry to resolve cell-type– and cell-cycle–dependent responses. Under identical conditions, mono-culture systems—particularly endothelial cells—exhibited amplified oxidative stress and inflammatory signaling with damage-associated markers. In contrast, tetra-culture showed attenuated inflammatory responses and no apoptosis markers, despite preserved antioxidant defense activation. While CeNP exposure promoted partial S-phase recovery in both systems, tetra-culture maintained a higher G0/G1 fraction and more stable baseline cell cycle architecture. Importantly, cell-cycle–resolved analysis demonstrated that S-phase recovery in tetra-culture occurred without activation of damage-associated markers. Overall, these findings show that mono-culture systems overestimate vulnerability to oxidative stress and CeNP exposure by activating canonical toxicity pathways. In contrast, multicellular culture complexity reveals that CeNP exposure suppresses lethal damage while reorganizing stress responses into a multicellular-adapted state, underscoring the importance of advanced, single-cell–resolved NAMs to improve the biological relevance and predictive value of respiratory nanotoxicity assessment.

P099
An AOP-based in vitro evaluation of humidifier disinfectant–induced pulmonary fibrosis using a 3D human epithelial–fibroblast co-culture model
ABSTRACT #373

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Pulmonary fibrosis is characterized by progressive extracellular matrix deposition and irreversible airway remodeling, primarily driven by dysregulated epithelial–mesenchymal interactions. Within the adverse outcome pathway (AOP) framework, epithelial injury represents an initiating event that triggers

downstream key events (KEs), including oxidative stress, inflammatory signaling, and fibroblast activation, ultimately leading to fibrotic tissue remodeling. Mechanistic evaluation of chemical-induced fibrogenesis therefore requires physiologically relevant human-based in vitro models capable of capturing these sequential events. In this study, we established a human-relevant 3D epithelial–fibroblast co-culture model to evaluate humidifier disinfectant (HD)–induced pulmonary fibrosis using a multi-endpoint, AOP-informed strategy. Human small airway epithelial cells (SAECs) were differentiated under air–liquid interface (ALI) conditions and validated by immunocytochemistry (ICC), hematoxylin and eosin (H&E) staining, and functional assessment of ciliary beat frequency (CBF). Differentiated epithelial layers were co-cultured with pulmonary fibroblasts using a transwell system to enable epithelial–mesenchymal crosstalk. Following acute exposure (3 h) to HD active components (PHMG-HCl, PGH, and CMIT/MIT), significant increases in cytotoxicity and intracellular oxidative stress were observed, representing early KEs. Epithelial dysfunction was evidenced by increased epithelial thickness and marked reductions in transepithelial electrical resistance (TEER), CBF, and ciliary beat coverage (CBC). Upregulation of transforming growth factor-β1 (TGF-β1) and pro-inflammatory cytokines indicated activation of profibrotic signaling pathways. Importantly, fibroblast-to-myofibroblast transition (FMT), a critical downstream KE associated with extracellular matrix accumulation, was induced in co-cultured pulmonary fibroblasts. Collectively, these findings demonstrate that HD exposure activates a coordinated sequence of AOP-relevant key events, linking epithelial injury to fibroblast activation and fibrotic remodeling. This 3D human-relevant co-culture platform provides a mechanistically anchored approach for AOP-based inhalation toxicity assessment.

P100
Short-term exposure to titanium dioxide nanoparticles induces transformation-associated phenotypes in human bronchial epithelial cells
ABSTRACT #374

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Titanium dioxide (TiO_2), widely used in industrial and consumer products, has been classified as possibly carcinogenic to humans (IARC Group 2B). However, its carcinogenic potential remains insufficiently characterized in human-relevant in vitro systems. In this study, we applied an adverse outcome pathway (AOP)-oriented integrated testing strategy (ITS) to evaluate the carcinogenic potential of TiO_2 using human bronchial epithelial BEAS-2B cells. TiO_2 exists in distinct crystalline phases, including anatase, rutile, and the mixed-phase P25, yet the influence of phase-dependent physicochemical properties on carcinogenesis-related responses remains unclear. We therefore investigated whether these TiO_2 forms induce phenotypic alterations associated with carcinogenic progression. Physicochemical properties were characterized using transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential analysis. Lung deposition was estimated using the Multiple-Path Particle Dosimetry (MPPD) model. Following short-term (24 h) exposure, TiO_2 induced reactive oxygen species (ROS) generation without significant cytotoxicity. Non-cytotoxic exposure ranges were established using crystal violet and colony-forming efficiency assays. Within these ranges, all TiO_2 forms significantly increased anchorage-independent colony formation. Cells derived from these colonies exhibited enhanced proliferative capacity. In addition, epithelial–mesenchymal transition (EMT)-related changes were observed, including decreased E-cadherin and increased N-cadherin expression, elongated morphology, and enhanced migratory capacity, with increased invasion under selected conditions. Collectively, these findings demonstrate that short-term exposure to TiO_2 nanoparticles, irrespective of crystalline phase, induces multiple transformation-associated phenotypes relevant to the promotion and progression

stages of carcinogenesis. This study supports the use of a multi-endpoint in vitro approach incorporating phase-specific properties for improved hazard assessment of TiO_2. Further investigations under long-term or repeated exposure conditions are warranted to better elucidate its carcinogenic potential.

P101
From primary NHBE cells to functional 3D inverted bronchial tissue: high-content imaging□based monitoring of differentiation
ABSTRACT #376

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Three□dimensional (3D) human bronchial epithelial cell models, cultured at the air–liquid interface (ALI) under inverted conditions, offer a physiologically relevant platform for respiratory research. These lung models recapitulate key structural and functional features of the airway epithelium, reflecting human-specific biology and therefore more predictive insights for respiratory toxicity testing than animal models or conventional 2D cultures. Despite their advantages, routine validation of epithelial differentiation still relies heavily on histology—an approach that is time-consuming, costly, and typically performed after experimental endpoints. As a result, insufficiently matured tissues may only be identified after study completion, risking loss of time and resources. To overcome this limitation, we investigated whether high content imaging (HCI), combined with functional readouts, could serve as a rapid and reliable method for longitudinal quality assessment. Inverted 3D bronchial cultures derived from primary Normal Human Bronchial Epithelial (NHBE) cells were maintained under ALI conditions and monitored weekly throughout differentiation. HCI was used to visualize key hallmarks of epithelial maturation, including tight junction formation, ciliation, the presence of mucus secreting cells, and the development of a pseudostratified, multilayered structure. In parallel, transepithelial electrical resistance (TEER) and ciliary beating frequency were measured to assess barrier integrity and functional activity. Conventional histology served as the reference standard. Our findings show that HCI, together with TEER and cilia□beating measurements, provides a sensitive and timely assessment of tissue maturation. Notably, HCI□derived structural and functional parameters showed strong correlation with histological outcomes. This integrated imaging based workflow reduces reliance on histology, lowers costs, and minimizes the risk of late detection of poorly differentiated tissues. By enabling earlier and more efficient quality control, it enhances the reliability of downstream studies and supports broader adoption of primary NHBE derived 3D bronchial models.

P102
Applicability of the soluairway™ human airway model as a NAMs platform for in vitro acute respiratory toxicity testing
ABSTRACT #384

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Acute inhalation toxicity assessments have traditionally relied on animal testing; however, such approaches are time- and resource-intensive and may not fully reflect human responses. Accordingly, the development of human-relevant in vitro non-animal alternative methods, known as Novel Approach Methodologies (NAMs), is increasingly required. In this study, an in vitro acute respiratory toxicity test method was established using SoluAirway™, a three-dimensional human airway tissue model derived from human nasal epithelial cells, and its applicability was evaluated using proficiency chemicals. SoluAirway™ is a human-derived tissue model that recapitulates the structural and functional characteristics of human

bronchial epithelium. Test substances (50 µL) were applied to the tissue surface and covered with a vapor cap, followed by exposure for 4 hours at 37 °C under 5% CO_2. After exposure, tissue viability was assessed using the MTT assay, and acute toxic responses were evaluated based on the results. Testing with proficiency chemicals demonstrated that the method produced stable and consistent response patterns, confirming its technical applicability and reproducibility. Furthermore, the test method showed the potential to evaluate toxic responses while reflecting the physiological characteristics of human airway tissue. The SoluAirway™-based test method represents a NAMs approach reflecting human relevance and has the potential to complement or replace animal testing for acute inhalation toxicity assessment. It is expected to serve as a reliable in vitro respiratory toxicity assessment platform for future regulatory toxicology applications. Key words: SoluAirway™, Acute respiratory toxicity, 3D model, Novel Approach Methodologies (NAMs), In vitro Funding Source: This work was supported by the Korea Environment Industry & Technology Institute (KEITI) through the Technology Development Project for Safety Management of Household Chemical Products Program, funded by the Korea Ministry of Environment (MOE) (grant number 202300230430).

P103
A human systemic model for simultaneously evaluating antibody-drug conjugates efficacy and the risk of interstitial lung disease
ABSTRACT #408

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Antibody–drug conjugates (ADC) development is limited by systemic toxicities, including interstitial lung disease (ILD). ILD is an inflammatory and fibrotic lung disorder that emerged as a safety concern for DXd-containing ADC, which is associated with diffusion to cells neighboring the target cell (bystander effect) and off-target toxicity upon entering circulation. Nonclinical models have limited ability to predict ADC-associated ILD, underscoring the need for human-relevant assays. We developed a human co-culture that combines an alveolar epithelial tissue model with encapsulated tumor spheroids, recapitulating alveolar–tumor crosstalk. The epithelium comprises primary human epithelial (type I and II pneumocytes) and endothelial cells cultured at an air–liquid interface, enabling physiologically relevant barrier and inflammatory responses. The tumor compartment uses encapsulated HER2+ SK-BR-3 spheroids. As clinical benchmarks, we evaluated T-DXd and free Exatecan. Since clinical ILD related to Trastuzumab emtansine (T-DM1) is rare, it was included as negative control. To probe the contribution of DXd-based payload chemistry across targets, we evaluated Patritumab deruxtecan (P-DXd), a HER3-targeting DXd ADC. Here, T-DXd, and free Exatecan displayed antitumoral activity alongside lung injury signals similar to P-DXd, supporting a payload-driven component of pulmonary risk. Importantly, the presence of tumor spheroids potentiates the lung toxicity induced by T-DXd and P-DXd, suggesting that the bystander effect contribute to the mechanism of lung toxicity. In alignment with clinical observations, T-DM1 did not induce injury signature to the lung. This integrated assay de-risks ADC programs by simultaneously quantifying tumor efficacy and lung safety in a human-relevant platform.

P104
Impact of berry-flavoured nicotine-free e-liquids on an in vitro model of the alveolar-capillary barrier: in vitro toxicity evaluation and chemical analysis
ABSTRACT #414

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Background/Objectives: Electronic cigarettes emerged on the tobacco-related products

market in the 2010s. Initially intended for smokers wishing to quit, they are particularly appealing to younger generations, notably due to the wide variety of flavours available. We previously have shown differences in term of toxicity depending on flavours and the method of exposition used: by dilution or exposure to trapped aerosol. Our aim here is to compare the toxicities of four berry flavours (Blackberry, Blueberry, Raspberry, Strawberry) on an in vitro model of the alveolar-capillary barrier (ACB) after direct exposure to aerosols and to link these effects to the chemical composition of each flavor. Material/Methods: An in vitro model of ACB, composed of the EA.hy926 endothelial cell line and the NCI-H441 epithelial cell line, was used to mimic the alveolar region. The alveolar compartment was placed in an Air-Liquide interface in order to be exposed, via a Vitrocell® device, directly to nicotine-free berry-flavoured aerosol (10 to 240 puffs). Several investigations were conducted at 24h, including barrier integrity by measuring TER, cell mortality by measuring extracellular LDH and enzymatic activity by MTS colorimetric assay. A qualitative and non-exhaustive chemical analysis was conducted on the four berry-flavoured e-liquids using TD-GC-MS in order to identify differences in aerosol composition. Results: An increase in TER, at different concentrations and varying degrees, was observed for the different flavoured e-liquids. Interestingly, this increase in TER was paired with a rise in cell death and enzyme activity, which were especially high for Blackberry and Blueberry flavours. Analysis by TD-GC-MS revealed significant differences between the chemical compositions of the various e-liquids, consistent with the cellular investigations conducted. Discussion/Conclusion: These results highlight the potential impact of flavourings in e-liquids and the need for further investigation into the wide variety of flavourings and their cellular effects.

P105
Lung surfactant inhibition assay - a method to predict early signs of acute inhalation toxicity
ABSTRACT #418

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Background Acute inhalation toxicity testing for regulatory purposes relies on the use of guideline studies in rodents. The results from acute inhalation toxicity testing are systemic (lethal concentration for 50% of the exposed animals) and death obscures effects on the portal-of-entry, i.e. the lungs. Understanding the effect on the lungs, is essential for replacement of animals for acute inhalation testing. However, the lung is a complex organ, with different potential targets for toxicity. Here the reliability and relevance of a cell-free method based on the monitoring of lung surfactant biophysical function during exposure to a test chemical for predicting acute effects on the lungs will be presented [1]. Method Measuring lung surfactant function addresses the molecular initiating event of the adverse outcome pathway AOP 302, the adverse outcome is “decreased lung function” [2]. The main function of lung surfactant is to regulate the surface tension at the respiratory air-liquid interface to avoid alveolar collapse in vivo, starting a cascade that leads to decreased lung function. The effect of more than 150 different chemicals and products have been tested, covering consumer products, occupational exposures, inhaled pharmaceuticals and single chemicals. Results The current applicability domain of the method will be explored, and how the results of this in vitro test relate to outcomes in guideline studies for acute inhalation toxicity [3]. Discussion and Conclusion This cell-free method is a promising candidate for prioritization and screening of chemicals, and its inclusion in an integrated approach to testing and assessment will contribute to the reduction of the use of rodents for acute inhalation toxicity testing. This work was supported by ‘FFIKA, Focused Research Effort on Chemicals in the Working Environment’ from the Danish Government.

P106
Mechanistic assessment of methyl methacrylate using human relevant new approach methodologies
ABSTRACT #422

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Methyl methacrylate (MMA) is a widely used industrial chemical for which respiratory sensitisation has been discussed despite limited mechanistic evidence. This study applied human relevant new approach methodologies (NAMs) to investigate whether MMA induces immune activation patterns consistent with respiratory sensitisation. MMA was evaluated in two complementary lung relevant NAMs: ALIsens®, a three dimensional human alveolar co culture model exposed at the air–liquid interface, and human precision cut lung slices (hPCLS). ALIsens® cultures were exposed to a non cytotoxic concentration of MMA (500 µg/cm²), followed by targeted gene expression analysis in dendritic like THP 1 cells. A predefined 20 gene panel was selected based on relevance to respiratory sensitisation mechanisms, covering innate immune sensing, antigen presentation, co stimulation, cytokine signalling, and intracellular pathways aligned with established adverse outcome pathway frameworks. In parallel, hPCLS from a human donor lung were exposed to MMA and reference substances, with assessment of gene expression, tissue viability, lactate dehydrogenase release, and CXCL8 secretion. In ALIsens®, MMA did not induce statistically significant or biologically relevant changes across the targeted gene panel. In hPCLS, MMA caused only a single statistically significant transcriptional change (GCSFR, ~1.7 fold), which was below commonly accepted thresholds for biological relevance and occurred in the absence of coordinated immune or inflammatory pathway activation or functional inflammatory responses. Importantly, exposure to LPS + TSLP induced the expected innate immune response, confirming tissue sensitivity and functional responsiveness of the hPCLS model. In contrast, the known respiratory sensitiser chloramine T trihydrate elicited a robust multi gene immune activation signature and clear functional effects, confirming assay responsiveness. These results consistently indicate that MMA does not trigger respiratory sensitisation like immune mechanisms under the conditions tested and demonstrate the value of targeted transcriptomic approaches in advanced human lung NAMs for mechanistic hazard assessment.

P107
Searching minimal coculture conditions to reproduce the initial inflammatory response to inhaled carbon nanoparticles
ABSTRACT #423

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Carbon-based nanomaterials (CBN), ranging from soot-like carbon nanoparticles (CNP) to carbon nanotubes (CNT), are increasingly used in industry, yet inhalation of these particles is linked to lung inflammation and interstitial lung disease. Prior single-cell RNA sequencing (Voss & Han et al., ACS Nano 2025) identified material- and cell-specific inflammatory circuits in vivo, but monocultures often fail to capture the multicellular crosstalk that drives these responses. Here we seek to identify the minimal coculture conditions, to model the previous detected inflammatory transcriptional responses of the alveolar niche, using mono-, co-, and triple-culture systems with MLE-12 (epithelial), J774.1 (macrophage), and CCL206 (fibroblast) cells. To apply equipotent doses, we used CBM concentrations causing 20 and 50% viability decreases within 24 h post-exposure (WST-1). qPCR at 6 h revealed particle- and cell-specific induction of the respective inflammatory mediators consistent with in-vivo profiles. Cxcl1 and Csf2, mapped to alveolar epithelial cells in vivo upon CNP exposure was best recapitulated in vitro by MLE-12/J774.1 co-culture model, indicating requirement of epithelial-macrophage interaction. For double walled carbon nanotubes (DWCNT) exposure, J774.1 cells were able to reproduce the

alveolar macrophages mapped Ccl3 gene expression, whereas the fibroblast mapped Ccl2 required co-culture with CCL206. Our models however failed to recapitulate the for MWCNT specific response of in fibroblasts induced Ccl11 and -19 expression in vivo. We hypothesize that the in the lungs detected release of the preformed alarmins (e.g. IL1a and IL33), which were transcriptionally not detected in the current in vitro models are essential for subsequent chemokine expression upon MWCNT injury. These findings demonstrate that multicellular systems are essential to reproduce the initial cytokine pattern triggered by CBN exposure. Aligning these in vitro responses with macrophage–epithelial–fibroblast circuits observed in vivo provides a mechanistic basis for physiologically relevant models in nanomaterial safety assessment NAMs.

P108
Impact of nicotine on calcium flux and markers of endoplasmic reticulum stress in vitro
ABSTRACT #437

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Background and Objectives Cigarette smoking constitutes a major public health issue, as the leading cause of respiratory diseases as Chronic Obstructive Pulmonary Disease (COPD). Tobacco's addictive properties stem primarily from nicotine, yet its specific effects on the alveolar capillary barrier remain poorly understood. Our research aims to explore the impact of nicotine on calcium flux and its consequences on endoplasmic reticulum (ER) stress markers in alveolar epithelial cells. Material and Methods Our in vitro alveolar model is based on the NCI-H441 cell line, which is a human alveolar type II cell line. To investigate calcium flux, cells were exposed to increasing concentrations of nicotine (0.0025 mM to 100 mM), digitonin (0.01%) as a positive control, and EGTA (5 mM) as a negative control using a fluorescent probe Fluo-4. Then, cells were treated with nicotine, N-acetylcysteine (5 mM) and tunicamycin (2 µg/mL) to study ER stress markers by Western blot: CHOP, BiP, Calnexin, PDI, ERO-1-α, IRE1-α/Phospho-IRE1-α, PERK/Phospho-PERK and EIF-2α/Phospho-EIF-2α. Results Intracellular calcium flux increased immediately after exposure to nicotine higher than 6.25 mM. The EGTA combination to nicotine treatment significantly reduced intracellular calcium flux. The expression of CHOP and Phospho-EIF-2α/EIF-2α increased dose-dependently after nicotine exposure, with results becoming statistically significant compared to the control at 6.25 mM of nicotine. Tunicamycin combined with increasing concentrations of nicotine strongly increases BiP and the rapport Phospho-EIF-2α/EIF-2α expression.

Discussion and Conclusion The results showed an increased intracellular calcium concentration after nicotine exposure that affects ER stress markers like CHOP. Further investigation is needed to clarify the impact of increased ER stress on cellular processes such as apoptosis.

P311
Gaining scientific confidence in determining respiratory tissue irritation of inhaled chemicals using in vitro approaches: from workshops to multi-laboratory studies
ABSTRACT #90

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Regulatory agencies worldwide have established requirements to evaluate the impact of inhaled chemicals on humans. Although inhalation toxicity testing has mainly been conducted on rats, differences in respiratory tract anatomy and biology between humans and rats limit the accuracy of predicting human effects [1]. This presentation will showcase our collaborative efforts from workshops to multi-laboratory studies with the goal of building scientific confidence and gaining regulatory approval for the use of an in vitro method to assess respiratory tissue

irritation. Respiratory toxicity was initially assessed for two surfactants—Triton X-100 and oleoyl sarcosine—under three conditions: 1) a single exposure in a human bronchial epithelial cell line (BEAS- 2B) and a reconstructed human respiratory epithelium (MucilAir), 2) a single exposure in a human-derived immortalized alveolar epithelial cell line (AXiAEC), and 3) repeated exposures in MucilAir. The findings from this study as well as other published case studies, have supported the design of a multi-laboratory evaluation of approximately 20 blinded chemicals on MucilAir tissues. To this end, 30 µL (100 µL/cm2) of test chemicals were applied onto the apical side of the MucilAir tissues for 24 hours. Cytotoxicity, cell viability, and transepithelial electrical resistance (TEER) were assessed. The study included three laboratories located in the US, UK, and India. Overall, dose-response relationships with varying points of departure were observed for most chemicals. Further analysis is ongoing to assess the inter-laboratory reproducibility. Once unblinded, the predictability of the in vitro method to determine whether a chemical may cause respiratory tissue irritation will be assessed.

Knowledge sharing and education

P109
The convener: A trade association's role in advancing animal-free science for chemicals safety assessment
ABSTRACT #132

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Through their relationships with industry, regulators, academics and NGO scientists, trade associations are perfectly positioned to promote education, adoption and use of animal-free safety assessment for chemicals. Following the UK's exit from the EU, establishment of UK chemicals regulatory frameworks presented a unique opportunity for the UK cosmetics trade association (CTPA), to intensify its activities towards the integration of non-animal New Approach Methodologies (NAMs) into relevant regulations with the ultimate aim of phasing out animal testing. Sitting within a network of industry scientists, sister associations, testing houses, NGOs, academia and Government regulators, CTPA convenes all relevant stakeholders in this area through regular events to ensure vital conversations and progress towards the common goal does not occur in isolation. From organising seminars and workshops, to contributing to Government strategies and policy, as well as publishing cross-industry industry position papers, CTPA has positioned itself as a UK leader in sharing knowledge and expertise in NAMs. Regulatory adoption cannot inspire change unless paired with education. In addition to bringing stakeholders together, CTPA pioneers the delivery of purely practical, case-study centred training on animal-free human health and environmental assessments. Industry and regulatory scientists learn how to use NAMs and Next Generation Risk Assessment (NGRA) to make safety decisions, gaining confidence in using innovative approaches in everyday risk assessments. Trade associations play a crucial role in bridging the gap between theoretical scientific advancement and practical implementation. Associations facilitate open and frank scientific dialogue between key partners, foster collaboration between all stakeholders, deliver original education and publicise key initiatives. The work of a trade association is therefore critical to progressing the phase- out of animal testing for chemicals regulatory purposes.

P110
Inova hub: illuminating the full landscape of NAMs
ABSTRACT #188

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The Netherlands was the first EU member state to develop a roadmap for phasing out the use of laboratory animals and stimulating the adoption of alternative models in scientific research. To facilitate and accelerate this transition, it is important to invest in the development of New Approach Methods (NAMs), such as organoids, organs-on-a-chip, and in silico techniques. For broad acceptance of these NAMs, it is essential to highlight success stories, recent developments, as well as provide education. To contribute to the

implementation and acceptance of animal-free methods we have developed the INoVA Hub. The INoVA Hub leverages data-driven text-mining strategies and generative AI to identify NAMs and corresponding outcomes in PubMed. The platform allows free text searches (NAM name and outcome) as well as filtering on characteristics of the described NAMs. These include, e.g. the type (e.g. in vitro) and format (e.g. organoid) of the NAM, but also the application domain of the described model (e.g. cardiovascular disease). The platform is updated on a daily basis to ensure researcher have access to the most recent information. By ensuring that (future) scientists can easily gain insight into available NAMs within a specific research setting, we aim to significantly contribute to the implementation and development of NAMs. The INoVA hub is freely accessible for non-commercial use through a web-based interface: inovahub.tno.nl. This work was funded by the ZonMW MKMD program.

P111
Science communication: bridging the gap between animal research and new approach methodologies (NAMs): a knowledge sharing campaign
ABSTRACT #256

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The integration of NAMs into biomedical research is critical for advancing ethical practices in life sciences. However, a gap exists between animal researchers and the broader NAM community. This abstract presents a knowledge-sharing campaign designed to bridge this gap by introducing NAMs, simplifying complex concepts with relatable analogies, and raising awareness of their benefits. The campaign aimed to increase interest in NAMs among animal researchers and promote adoption of alternative methodologies. Structured around five content pillars—NAM Definition and Importance, Types, Benefits, Challenges and Solutions, and Success Stories—the campaign featured three strategically timed LinkedIn posts per pillar. Targeting key organizations such as EARA, FELASA, and EAAP, posts engaged their established communities to ensure relevant reach. Peak user activity periods (14:00 CET, Wednesdays) were used to maximize visibility. Content drew on the latest scientific literature and employed analogies to make NAM concepts accessible to animal researchers. Effectiveness was assessed via mid- and post-campaign evaluations, including a poll and Q&A session. Metrics indicated strong engagement: Altertox's LinkedIn page gained 139 followers on campaign post days, posts averaged 667 impressions, and click-through rate reached 4.5%. A poll revealed 100% of respondents correctly identified NAMs' key benefits over traditional animal testing, demonstrating successful knowledge transfer. This campaign highlights the potential of strategic social media outreach to bridge gaps within life sciences and integrate NAMs into research curricula and training programs, fostering a culture of responsibility, ethics, and innovation. Findings underscore the importance of raising awareness and promoting NAM use, aligning with the 3Rs (Replace, Reduce, Refine) principles and advancing ethical scientific practice. Communication campaigns can effectively promote NAMs and provide insights for integrating these methodologies into scientific education, supporting a more informed, responsible, and ethically engaged research community.

P112
The potential protection of Tamarix extracts against toxicity induced by selected beauty products in Hs27 human skin fibroblasts.
ABSTRACT #289

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Abstract: The current study evaluates the cytotoxicity and chemical analysis of selected beauty products and evaluation of the protective effect of Tamarix articulata (TA) extract against toxicity induced by beauty products in skin fibroblasts (Hs27). MTT and Crystal violet (CV) assays were used to determine the dose-dependent cytotoxic effects of products against Hs27 fibroblasts. DNA

fragmentation assay was conducted to determine the mode of cell killing induced by evaluated beauty products. Chemical analysis and heavy metals were evaluated to determine beauty products. Pre-treatment with TA extract for different time points followed by time-dependent exposure with beauty products to assess the protective effect of TA extract in Hs27 cells was analysed by MTT and CV assays. Owing to the presence of various harmful heavy metals such as arsenic (As), chromium (Cr), cadmium (Cd), nickel (Ni), and lead (Pb) in beauty products, our results revealed that all beauty products induce significant cytotoxicity over time (1, 4 h) in a dose-dependent (125, 250, 500 µg/mL) manner. DNA fragmentation assay revealed that the induced cytotoxicity was caused by necrosis. However, pre-incubation with a safe dose (50 µg/mL) of TA for different times (24, 48 h) followed by exposure to various doses (62.5, 125, 250, 500 µg/mL) of beauty products for different times (1, 4 h) revealed significant (*p≤0.05, **p≤0.01) protection against product-mediated cytotoxicity. The effect was more pronounced for 1 h exposure to products compared to 4 h. Our study demonstrates that the due to the presence of heavy metals in synthetic beauty products exhibits marked toxicity to skin fibroblasts. However, the presence of abundant bioactive polyphenols with promising antiscavenging activity in TA extracts significantly nullifies cytotoxicity promoted by examined beauty products in skin fibroblasts (Hs27). Keywords: Tamarix, beauty products, cytotoxicity, Hs27 cells, bioactive compounds.

P113
Re-place: a national platform to strengthen dissemination and uptake of new approach methodologies in Belgium
ABSTRACT #323

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Advances in New Approach Methodologies (NAMs) such as in vitro methods, in silico models and in chemico assays have contributed to a shift in toxicology towards more mechanism-based and human-relevant safety assessment. While similar but diverse approaches are emerging across biomedical research, toxicology remains the discipline in which NAMs are most formally integrated into regulatory frameworks. As the field of NAMs is continuously evolving, identifying relevant, reliable and up-to-date information about their practical applications remains an important challenge for many stakeholders. To address this need, the RE-Place platform was launched to collect and promote expertise on NAMs available in Belgium in one central open-access database (www.re-place.be). The database features methods submitted directly by scientists, for which the name and organisation of the contributor are explicitly indicated, providing a direct point of contact for other scientists seeking further information on specific NAMs of interest. In January 2026, the RE-Place database contained 334 methods added by 217 experts from 35 Belgian organisations. These methods cover different areas from basic and applied research to regulatory use and education, and approximately a third of them is related to the field of toxicology. The majority of expertise present in the database concerns in vitro methodologies, including stem cell-based models, organoids, and microphysiological systems. Over the years, the RE-Place platform has continued to expand by actively inviting Belgian scientists to share their models in the database. As such, contributors can benefit from increased visibility through the database, as well as through targeted dissemination and communication activities including the newsletters, social media and RE-Place educational webinars. By promoting knowledge exchange, RE-Place enhances collaborations among researchers, regulators, industry, competent authorities and other stakeholders. This collective effort accelerates the broader adoption of NAMs in toxicology and other fields of life sciences, supporting the long-term transition toward animal-free research.

P114
ICCS best practice guidance document for serious eye damage and eye irritation assessment using new approach methodologies for

substances in cosmetics and personal care products

ABSTRACT #396

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The transition toward modern safety assessment requires more than just New Approach Methodologies (NAMs); it requires a harmonized framework for data interpretation and decision-making. Following the successful launch of the International Collaboration on Cosmetics Safety (ICCS) Best Practice Guidance (BPG) for Skin Sensitization in 2025, there is a clear need for a parallel resource for serious eye damage and eye irritation. The objective of this work was to develop a comprehensive and harmonized document that provides a structured, science-based workflow for assessing serious eye damage and eye irritation without the generation of new animal testing. This BPG aims to equip safety assessors with the tools to navigate Organisation for Economic Co-operation and Development (OECD) test guidelines and non-OECD methods and approaches, integrating them into a robust Next Generation Risk Assessment (NGRA) framework. Collaboratively developed by over 20 industry experts and toxicologists spanning Canada, the EU, Japan, Latin America, UK, and the U.S., the BPG presents a stepwise framework covering problem formulation, exposure context in a finished product, substance characterization, data collection, weight of evidence evaluation, targeted NAM testing, hazard determination, uncertainty analysis, and risk conclusion. It provides detailed descriptions of available NAMs, their applicability domains, and their integration into Defined Approaches (DA) inclusive of DAL 1 (defined approach for neat non-surfactant liquids), DAL 2 (defined approach, for non-surfactant neat liquids, liquids, and solids dissolved in water), DAS (defined approach for solids), and DASF (defined approach for surfactants). It also explores the exposure rationale for assessing substances at use concentration in a finished product. Designed for safety assessors transitioning from traditional animal models to modern approaches, this BPG reflects up-to-date scientific consensus, aligned with international regulatory expectations. The ICCS BPG for Serious Eye Damage and Eye Irritation Assessment is expected to be published in summer 2026.

P115

Structured research, adequate reproducibility: a digital approach to managing the complexity of in vitro studies

ABSTRACT #425

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In the context of the accelerated shift from animal testing to new approach methodologies (NAMs), in vitro models have become central to biomedical research. While witnessing their ever-growing adoption worldwide, it is important to recognize that the intra- and inter-laboratory reproducibility of cell-based studies can be challenging, as several factors such as biological variability, poor reagent quality and experimental inconsistencies can substantially limit assay replication. Moreover, as the field broadens towards advanced 3D in vitro models with higher physiological relevance, the experimental complexity and risk of variability increase significantly. One major contributor to assay variability is the incomplete reconstruction of experiments, as the exact circumstances (e.g., cell line characteristics, culture conditions, experimental setup, etc.) associated with assay variability are often recorded across multiple systems (e.g., laboratory notebooks, spreadsheets, files, etc.). To overcome this issue, a new digital approach that enables the documentation and real-time monitoring of 2D and 3D in vitro experimental conditions, is presented. The INVITRIO SCIENTIFIC platform was used to electronically monitor cell line details (e.g.,

identity, passage, type, source, etc.) and culture conditions (e.g., reagents, routine culture activities, confluence, etc.), followed by a consistent documentation of the spheroid formation parameters (e.g., seeding density, plate type, method, etc.) and features (e.g., morphology, size, integrity, etc.). The workflow was structured as a connected record (i.e., 2D model - 3D model - study - experiments - results), enabling the identification of potential variability sources. By integrating the complete in vitro workflow in a centralized system, this approach facilitates the accurate reconstruction of experiments, supports the obtainment of consistent results, and improves reproducibility.

P327
Conceptual framework using weight-of-evidence incorporating new approach methods for risk assessment
ABSTRACT #454

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Weight of Evidence (WoE) analysis supports hazard and risk assessment while reducing animal testing by supporting the integration of New Approach Methods (NAMs). A globally harmonized WoE framework, grounded in OECD's key principles, can foster broader adoption of NAMs. This can be achieved by establishing best practices for implementing the core principles of problem formulation, data collection, evidence evaluation and weighing, and evidence integration and reporting. In practice, these principles must be flexible to fit into regulatory contexts and support integrating various data types. For example, when working with in vitro data, it is important to emphasize assay validity, reliability, and reproducibility, whereas integrating read-across data requires a focus on structural similarity, consistency, and concordance. Therefore, WoE as a conceptual framework can only be applied as 'fit-for-purpose'. This case example applies a globally harmonized WoE framework to the human health evaluation of a chemical metabolite, implementing a NAMs approach to avoid unnecessary animal testing. Data collection included Threshold of Toxicological Concern (TTC) approach with QSAR analysis for Cramer classification and structural alerts; chemical analog identification for ID of structural analogs; comprehensive field trial residue data; and dietary exposure modeling. QSAR modeling and structural analogs indicated no alerts for genotoxicity, and the structure did not indicate potential for in vitro or ex vivo bioactivity. The metabolite was classified as Cramer Class III, with dietary exposure at <1% of the TTC. Based on the WoE, the metabolite was not included in the residue definition, and no additional animal testing was required. This case study illustrates the successful application of a WoE framework to a common regulatory scenario, providing a solid basis for safety assessment while promoting NAMs. It demonstrates how systematic evidence collection, evaluation, weighing and integration can inform decisions, minimize animal testing, and uphold scientific rigor in pesticide evaluations.

P328
The aspis academy: empowering the next generation of toxicologists
ABSTRACT #458

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In the context of a global shift toward animal-free toxicology, early-stage researchers (ESRs) play a central role in driving innovation through New Approach Methodologies (NAMs). The ASPIS Academy (AA) is a dedicated ESR network established in 2023 within the ASPIS cluster, which unites three Horizon 2020 projects: ONTOX (1), RISK-HUNT3R (2), and PrecisionTox (3). The AA plays a pivotal role in advancing animal-free chemical risk assessment by equipping over 120 ESRs with the skills and opportunities needed to innovate in the field of toxicology. Through a diverse portfolio of initiatives, including tailored training, mentoring, lab exchange (twinning) opportunities, and science communication, the AA fosters both scientific and transferable skill development. The Academy also embraces diversity and inclusivity, ensuring that researchers from all backgrounds can actively contribute to shaping the future of toxicology. Led by ESR representatives and supported by senior scientists, the AA fosters a dynamic environment for knowledge exchange and peer support across disciplines. It empowers its members to engage with NAMs in vitro test systems, in silico tools, omics technologies, and artificial intelligence, preparing them to lead in the transition toward human-relevant, non-animal testing strategies. This poster presents the ASPIS Academy as a model for ESR engagement and capacity-building and aims to open dialogue with other networks to explore synergies, shared challenges, and collaborative opportunities in shaping the next generation of toxicologists.

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Local toxicity testing (safety and efficacy)

P116
Optimizing keratinocyte spheroid formation: efficiency and morphological outcomes
ABSTRACT #106

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Three-dimensional (3D) cell culture has the potential to revolutionize approaches to the development and validation of reliable in vitro methods. The technique employed in the present study primarily aims to adapt previously established assays developed in two-dimensional (2D) culture systems to the reality of three-dimensional (3D) cultures, following the guidelines set forth by the OECD, aiming to produce viable and reproducible spheroids for efficacy and safety testing, and to compare the cellular responses between two 3D culture techniques. Also, the goals are to analyze spheroid formation kinetics in plates from different commercial brands, as well as their morphology through confocal microscopy. Spheroids were generated using two distinct techniques. The first involved Ultra-Low Attachment (ULA) plates, referred to herein as the Self-Assembly method. The second utilized magnetic levitation. Both methods employed HaCaT and KeratinoSens™ cell lines. The comparison of 3D culture techniques, initially using the HaCaT cell line in cytotoxicity assays with selected OECD-proficient chemicals, revealed that cells within the spheroid model exhibit distinct behavior, showing increased resistance to the standard compounds compared to 2D models. This suggests that 3D spheroids accurately replicate in vivo conditions. Confocal microscopy with various fluorophores allowed detailed morphological analysis of spheroids formed by both HaCaT and KeratinoSens™ cell lines. The magnetic levitation method offered more practical handling of spheroids; however, it was more costly and proved incompatible with KeratinoSens™ cells, although it was successful with HaCaT cells. Conversely, the Self-Assembly method proved to be simple, cost-effective, and easily reproducible, making it a promising alternative for a wide range of

experimental applications, including the sensitization test OECD 442D (2024).

P117
Fruits from Brazilian Cerrado as a potential source for application in cosmetics: in silico and in vitro analysis
ABSTRACT #121

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Growing consumer awareness regarding the environmental impact and sourcing of cosmetic products has fueled a demand for natural and sustainable ingredients. The Brazilian Cerrado, a biodiversity hotspot, presents a rich and largely unexplored reservoir of such resources, particularly exotic fruits. This study focuses on the in silico analysis of bioactive molecules presents in six Cerrado fruits: Pequi (Caryocar brasiliense), Cajuzinho (Anacardium humile St. Hill), Baru (Dipteryx alata), Buriti (Mauritia flexuosa), Jatobá (Hymenaea courbaril L.), and Mangaba (Harconia speciosa), to evaluate their potential application in the cosmetic industry. This research revealed that the most prevalent molecules were carotenoids, vitamin C, and vitamin E, all of which demonstrated antioxidant activity. Furthermore, the in silico results were compared with in vitro experimental data regarding the cytotoxicity of different parts of the fruits. Computational assessments enabled the evaluation of the safety of the most abundant molecules found in the extracted parts, which were also used in the experimental tests. Based on this, it was possible to compare the hazard level of each fruit part with the sensitization potential of its most representative molecules, as indicated by the software used in silico analysis. Two different computer programs were used to perform the analysis, the PredSkin for skin sensitization, and ADMET Predictor®, for permeation and sensitivity. Results at ADMET Predictor® showed molecules evaluated are sensitizers of murine skin through the LLNA (Local Lymph Node Assay) test. However, according to HRIPT (Human Repeated Insult Patch Test) parameter on PredSkin, β-carotene and ascorbic acid are not sensitizers of human skin. In silico analysis offers a powerful alternative to animal testing, unlocking the potential of biodiversity for sustainable innovation and economic growth, and when used in conjunction with in vitro analysis, can predict the potential risk of these important new raw materials.

P118
Nanocellulose as a food additive: assessing the toxicological implications of nanocellulose in the gastrointestinal tract.
ABSTRACT #125

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Research interest in the use of Nanocellulose (NC) as a functional material has been rising in recent years. Of the many different fields in which NC can be applied, its use as a food additive presents great potential as it is capable of modulating nutritional uptake with benefits [1]. Based on FDA guidelines, micron-sized cellulose is classified as a GRAS (Generally Regarded As Safe) material approved in food manufacturing, while NC is not. This is because bulk sized cellulose is considered to be non-toxic when consumed, but the same cannot be said for NC due to their potential uptake by the intestinal epithelium [2]. Regardless, current literature findings into the toxicity of NC as a food additive remain limited for it to be considered as a safe food ingredient. In this project, the toxicity profile of NC consumption will be evaluated using Caco-2 cells. NC was first added to a Standardized Food Model (SFM) [1] that is based on the US diet. The INFOGEST digestion model [3] was used to carry out simulated digestion of the food mix, where the digesta was quantified for changes in nutritional profile before exposure to the cells for toxicity evaluation. Quantification of

nutrients in the food matrix revealed that incorporation of NC reduced the overall digestion of lipids, proteins and carbohydrates. Subsequently, the exposure of pristine NC to Caco-2 cells resulted in a slight increase in oxidative stress to the cell, especially for smaller NC sizes. These findings indicate the potential of NC as a sustainable and non-toxic functional food additive.

P119
Comparison of in vitro skin sensitization strategy using a data set of cosmetic raw materials
ABSTRACT #147

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Allergic contact dermatitis is an inflammatory skin condition caused by repeated exposure to sensitizing chemicals. With the European Cosmetic Regulation 1223/2009 ban on animal testing, alternative approaches are required to assess skin sensitization potential. A study conducted by Clouet et al. (2017) aimed to compare and validate an integrated in vitro assay strategy based on three complementary tests representing the first three key events in the adverse outcome pathway of skin sensitization: DPRA, SENS-IS, and h-CLAT. A dataset of 33 known sensitizers was used to evaluate the predictive performance of these tests individually and in combination. Results were interpreted using a Weight-of-Evidence approach with a “2-out-of-3” rule: a chemical is classified as a sensitizer if two of the three assays are positive. Among the different combinations, the optimal testing sequence identified was DPRA + SENS-IS, followed by h-CLAT if results were equivocal. To confirm this sequence, an analysis of results obtained on 224 cosmetic raw materials was conducted. By comparing the different combinations, the h-CLAT + SENS-IS pair provided the best result with identical classification in both tests (158/224) compared to the DPRA + SENS-IS and DPRA + h-CLAT pairs. This optimal sequence h-CLAT + SENS-IS followed by DPRA, differs from that previously on reference molecules. This new analysis highlights the importance of the sample analyzed in terms of both size (224 vs. 33) and variety. Unlike the study by Clouet, the majority of raw materials tested were mixtures of compounds of varying nature (fragrances, natural extracts, etc.). While all 33 compounds were sensitizers, not all cosmetic raw materials were. Moreover, the sample analyzed contained a high proportion of perfumes (103/224), which could influence the test results. These observations could explain the difference with the sequence previously described.

P120
An in vitro strategy using RhE to assess the tolerance and efficacy of personal care products based on a concrete example: a repair balm
ABSTRACT #162

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The development of personal care products has traditionally relied on in vivo testing in human volunteers to evaluate tolerance and efficacy. In recent years, in vitro approaches have emerged as valuable complementary tools—not only for ethical considerations but also to generate mechanistic data that would otherwise be invasive in humans. Such approaches provide insights into the biological pathways involved and contribute to optimizing product performance. Among these, Reconstructed Human Epidermis (RhE) models are widely used in cosmetic research. Beyond their established regulatory role in safety assessment, RhE-based assays are increasingly implemented throughout R&D phases to evaluate both product tolerance and efficacy. These reconstructed skin models closely mimic the structure and functions of native human epidermis, making them suitable for studying ingredients, ingredient mixtures, and complex formulations. This poster presents an in vitro strategy for the development of a cosmetic repair balm. Skin tolerance was first assessed through cell viability assays and the expression of 20 inflammatory markers, taking into account environmental factors (the exposome) known to induce skin damage and potential product toxicity. The product’s reparative efficacy was then evaluated based

on tissue morphology, barrier integrity (trans-epithelial electrical resistance), and the expression of key proteins involved in proliferation (Ki67) and differentiation (filaggrin) over a 7-day period. Results were compared with those of commercially available reference products and revealed distinct repair mechanisms, highlighting the value of in vitro reconstructed skin models in guiding cosmetic product development.

P121
Epidermal repair after superficial burn: in vitro evaluation of reparative effects and soothing products
ABSTRACT #163

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Epidermal repair following a burn injury is crucial for maintaining the integrity and function of the skin barrier. Numerous cosmetic products have been developed to accelerate skin recovery after burns. However, for ethical reasons, it is not possible to assess the effectiveness of such products on volunteers with skin injuries within the field of cosmetics. In vitro methods provide an alternative that allows data collection without resorting to invasive techniques on humans. This data, obtained using reconstructed human tissue models, is valuable for understanding the mechanisms of action and improving the reparative effects of products. This poster presents the implementation of a method for evaluating the reparative effect on skin damaged by a superficial burn, as well as the testing of soothing products. To achieve this, an in vitro model of reconstructed human epidermis (AlternaSkin®) was utilized. The first step involved inducing a superficial burn in a reproducible manner, by precisely defining experimental parameters such as instrument choice, temperature, and contact time. The next phase involved identifying appropriate endpoints to assess the effectiveness of products in repairing the epidermis after such injury. Parameters chosen for evaluation included cell proliferation, barrier function assessed via trans-epidermal electrical resistance (TEER) measurements, tissue morphology using hematoxylin-eosin staining, and the expression of filaggrin, a protein involved in the formation of the epidermal stratum corneum, via immunostaining. The results indicate that certain parameters are more relevant than others for studying skin repair, with the area surrounding the burn site proving to be the most suitable for evaluating the reparative process. The tested products showed a significant improvement in both morphology and filaggrin production. This experimental protocol appears to be well-suited for studying skin repair following a superficial burn injury.

P122
Evaluation of human blood as a sustainable alternative to bovine blood in the red blood cell (RBC) test for eye irritation assessment
ABSTRACT #176

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The Red Blood Cell (RBC) test is a well-established in vitro method for evaluating the eye irritation potential of cosmetic ingredients and formulations. Traditionally, bovine blood (a by-product of the meat-industry) as a source of erythrocytes has been used due to its high reproducibility. However, ethical and sustainability considerations have driven the search for animal-free alternatives. This study explores the use of human blood, that is no longer suitable for transfusion, as a replacement for bovine erythrocytes in the RBC test. Hemolysis and protein denaturation were quantified, and their ratio was calculated to assess irritation potential. A panel of reference substances was tested to compare the performance of human versus bovine erythrocytes. Results demonstrated that human blood provides consistent and reproducible outcomes, with no statistically significant influence observed so far from donor age, blood type, or sex. These findings support the suitability of human blood as a robust and physiologically relevant alternative to bovine blood in the RBC test. Being physiologically closer to humans, the use of human blood may enhance the predictive accuracy of the RBC test and support the transition toward more human-relevant in vitro models in toxicology. This work supports the cosmetics industry's

commitment to not only comply with the ban on animal testing, but also to provide animal derived-free in vitro assays wherever possible and in accordance with regulatory guidelines.

P123
Investigation of the intestinal effects of dietary advanced glycation endproducts in an vitro model of the inflamed intestine
ABSTRACT #191

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Advanced glycation endproducts (AGEs) are a heterogenous group of compounds formed in the Maillard reaction, a non-enzymatic browning reaction between carbohydrates and proteins. Endogenously produced AGEs are involved in aging, inflammation, and intestinal barrier impairment. However, the effects of dietary AGEs on inflammation remain insufficiently characterized. Moreover, questions remain regarding their impact on ongoing intestinal inflammation as well as the influence of different Maillard reaction model systems (MRPs). Five different sugar/protein (100 nM and 5 g/L, respectively) MRPs (i.e. fructose/soy, fructose/lentil, fructose/whey, lactose/casein and fructose/casein) were prepared in glucose-free DMEM and either used without further treatment (t0) or heated at 100°C for 120 min (t120). An intestinal triple culture model was established using Caco-2, HT29-MTX-E12 and PMA-differentiated (100 nM, 24h) THP-1 cells. An inflamed-like state was induced using LPS and IFNγ (10 ng/mL each) [1]. The triple culture model was exposed apically to both t0 and t120 samples at concentrations of 33% (v/v) for 24h and analysed for barrier integrity (transepithelial electrical resistance), cytotoxicity (LDH assay), and cytokine secretion (ELISA). The inflamed state was characterised by various hallmarks of intestinal inflammation, including barrier disruption, necrotic cell death and increased release of pro-inflammatory cytokines. All five AGEs induced distinct, composition-dependent effects. Enhanced release of mucus was observed after exposure to fructose/lentil (t0, t120), lactose/casein and fructose/casein (both t120). Enhanced cytotoxicity was observed after exposure to lactose/casein (t120) and fructose/lentil (t0 and t120). In line with this, barrier integrity was impaired in cultures exposed to lactose/casein (t120) and fructose/lentil (t0). The results suggest an enhanced susceptibility of inflamed intestinal tissue to dietary AGE exposure. Furthermore, the observation of composition-specific effects of the investigated AGEs highlights the importance of the biochemical characteristics of AGEs, which warrants further research into their potential role in intestinal inflammation.

P124
Integrated transcriptomics as a new approach methodology (NAM) for predictive chemical safety and efficacy assessment in skin models
ABSTRACT #201

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The transition toward New Approach Methodologies (NAMs) demands innovative, mechanistically grounded tools to evaluate compound safety and efficacy without animal testing. Transcriptomics provides a powerful foundation for such approaches, by capturing the full spectrum of molecular responses induced by chemicals in human-relevant systems. Here, we present an integrated, multi-scale transcriptomic framework combining bulk RNA sequencing (SmartOmic™), mechanistic scoring (OmicSkin™), and single-cell deconvolution (SkinMap™) to support predictive and quantitative NAMs for evaluating skin responses to cosmetics or drugs, both at the gene expression and cellular resolution levels. SmartOmic™ leverages BRB-seq, a cost-efficient bulk RNA-seq technology optimized for high-throughput applications. Applied to reconstructed human epidermis and keratinocyte cultures, it enables robust and reproducible expression profiling across dose-response and time-series experiments. The resulting profiles are analyzed through OmicSkin™, a standardized scoring system that integrates eight mechanistic endpoints to convert transcriptomic data into interpretable

biological insights: including inflammation, irritation, and sensitization for safety; anti-aging, pigmentation, detoxification, hydration, and skin regeneration for efficacy. To contextualize bulk signatures, SkinMap™, a single-cell meta-atlas of human skin (23 public studies, 1,380,090 cells) is used for computational deconvolution, bridging bulk and cellular resolution to predict cell-type–specific responses in models lacking single-cell data. Altogether, this integrated approach demonstrates how multi-scale transcriptomics enables NAMs by linking molecular responses to cellular mechanisms. By combining scalability, reproducibility, and human relevance, it paves the way for predictive, regulatory-aligned frameworks for cosmetic and dermatological innovation.

P125
Further development of the in vitro photoassay using THP-1 cells to identify phototoxic chemicals
ABSTRACT #250

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Background and objective: Photoallergic contact dermatitis arises when certain chemicals become photoactivated by light, eliciting immune-mediated skin reactions. Although no validated assays currently exist for assessing photosensitization potential, different in vitro models have been described in recent years. The use of THP-1 cells to discriminate between photoirritants and photoallergens is focused on the dendritic activation key event. This study aims to analyze the light sensitivity of cells associated with passage number and to compare the use of two different assays for assessing (photo)cytotoxicity. Material and methods: THP-1 cells were exposed to different concentrations of phototoxic compounds and non-phototoxic chemicals then irradiated with 2.5 J/cm2 UVA or kept in the dark. After 24 h, cytotoxicity was determined by the Lactate dehydrogenase and MTT assays. Non-treated cells under dark and irradiated conditions were used as controls. Cell viability was calculated for each sample against the corresponding untreated cells. THP-1 light sensitivity was controlled across different passages (4-28). Eighty and fifty percent cell viability (CV80; CV50) was calculated for each chemical by linear regression analysis of the data. Results: The mean viability and standard deviation of control cells exposed to light was 105.3 ± 2.5 for the LDH method and 96.4 ± 4.5 for MTT. No association was found between lower values of viability and cell passage number. In general, the CV80 and CV50 calculated for the phototoxic chemicals studied in light conditions were lower than in dark conditions while similar values were obtained by the non-phototoxic chemicals. Ongoing studies determining the IL-8 release by THP-1 when exposed to non-toxic concentrations would help to discriminate between photoirritants and photoallergens. Discussion and conclusion: The use of THP-1 as a model to identify phototoxic chemicals provides a promising, cell-based alternative for evaluating the photosensitizing potential of chemicals and supports next-generation, animal-free risk assessment strategies.

P126
Empowering discovery of safe and effective drugs targeting cystic fibrosis infections by using new Candida albicans-CF zebrafish model
ABSTRACT #297

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Cystic fibrosis (CF) predisposes patients to chronic fungal colonization, particularly by Candida albicans, which adapts to the altered mucus microenvironment and triggers

sustained inflammation. Clinical antifungal drugs often fail to eradicate Candida due to its ability to persist in biofilm forms, while the expression of virulence factors completely remained unknown. Our aim was to establish a zebrafish CF infection model to study C. albicans pathogenicity and to identify novel bioactive compounds that selectively target fungal virulence rather than growth. CFTR-deficient zebrafish embryos were generated by antisense oligonucleotide knockdown. Larvae were microinjected with GFP-expressing C. albicans strains, including SC5314 and clinical CF isolates. Fungal burden, morphology, and host toxin - candidalysin expression were monitored using fluorescence microscopy. A library of synthetic and natural bioactive molecules was screened in vivo for antifungal efficacy, toxicity, and modulation of ECE1 expression. CF zebrafish exhibited enhanced susceptibility to infection, characterized by persistent pseudohyphal growth and elevated ece1 expression compared to wild-type controls. Several tested compounds inhibited filamentation and ECE1 expression without affecting fungal viability. These molecules significantly reduced tissue inflammation and improved embryo survival. None displayed detectable toxicity in the zebrafish model, supporting their favorable safety profile. The zebrafish CF model proved to be a powerful platform for dissecting the virulence dynamics of Candida albicans under CF-like conditions. We demonstrate that selective inhibition of virulence pathways, rather than fungicidal activity, effectively attenuates infection severity. The newly identified bioactive molecules represent promising leads for antivirulence therapy development in fungal infections associated with CF. This study highlights the translational value of zebrafish for preclinical discovery of safe and effective antifungal strategies.

P127
Multimodal neurotoxicity profiling of pesticides using a human iPSC-based organ-on-chip platform
ABSTRACT #299

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The development of alternative methods to assess pesticide-induced neurotoxicity is essential for improving safety and reducing reliance on animal models. This project represents a collaboration between NETRI and ANSES to establish a humanized in vitro model combining human induced pluripotent stem cell (hiPSC)-derived neurons with NETRI's Dualink organ-on-chip platform. This system enables the investigation of pesticide effects on neuronal function in a controlled, physiological environment. In this proof-of-concept, we are utilizing two model neurotoxicants: rotenone and vanillin. Rotenone, a well-known pesticide, induces neurodegenerative processes similar to Parkinson's disease, while vanillin is considered a negative control. Both substances are tested on the in vitro platform to evaluate their impacts on neuronal activity, and toxicity markers. Dose-effect relationship will be first investigated to allow the selection of pertinent dose regiment for subsequent experiments. Multimodal data are collected through multiple techniques, including microelectrode array (MEA) recordings to assess neuronal electrical activity, and enzyme-linked immunosorbent assays (ELISA) for quantifying biomarkers related to neurotoxicity. By integrating these data types, we aim to create substance-specific Digital Signatures, providing a comprehensive view of their neurotoxic profiles. These signatures aim to differentiate between toxic and non-toxic compounds, thereby enhancing the predictability of neurotoxic events. This project represents a step forward a high throughput screening for pesticide-induced neurotoxicity, with the aim of providing to the community a comprehensive database of digital signature of neurotoxic pesticides.

P128
Development and optimization of an MCTT HCE™-based eye hazard identification assay
ABSTRACT #320

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Alternative in vitro methods have increasingly been developed and accepted for the identification of chemicals inducing serious eye damage or eye irritation, supporting the replacement of animal testing. The SkinEthic™ Human Corneal Epithelium (HCE) Time-to-Toxicity test, including protocols for liquids and solids, was adopted as OECD Test Guideline (TG) 492B in 2022. This guideline describes an in vitro approach enabling the identification of chemicals that do not require classification (No Category), require classification for eye irritation (Category 2), or require classification for serious eye damage (Category 1) according to the United Nations Globally Harmonized System (UN GHS). To address the growing need for alternative test methods applicable to Good Laboratory Practice (GLP) facilities in Korea, the MCTT HCE™ Eye Hazard Identification Assay (EHA) was developed and optimized using a domestically produced reconstructed human cornea-like epithelium (RhCE). The assay was designed as a me-too approach aligned with the principles of OECD TG 492B while reflecting the specific characteristics of the MCTT HCE™ model. A standardized operating procedure (SOP) compatible with TG 492B was established to ensure methodological consistency. The predictive performance of the assay was evaluated using two protocols, EHA-L for liquids and EHA-S for solids, by testing 37 liquid and 37 solid chemicals. Optimization focused on parameters such as test chemical concentration, applied dose, and washing procedures. The results demonstrate that the optimized method shows promising predictive capacity and supports its potential inclusion within OECD TG 492B. A multi-laboratory validation study using the optimized protocol is planned to further confirm reproducibility and robustness. Keyword: Eye hazard identification assay, MCTT HCE™, RhCE, KoCVAM Funding Source: This research is supported by a grant (25211MFDS001) from Ministry of Food and Drug Safety.

P129
Establishment of a KeraSkin™ RhE-based skin sensitization test method
ABSTRACT #321

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The KeraSkin™ Skin Sensitization Assay is a keratinocyte activation-based in vitro method using reconstructed human epidermis (RhE) to support skin sensitization assessment. The assay quantifies the expression of four marker genes (ATF3, IL-8, DNAJB4, and GCLM) using real-time quantitative PCR (RT-qPCR), reflecting activation of the P2X7 receptor and Nrf2 signaling pathways. EpiSensA, adopted as OECD Test Guideline (TG) 442D, was used as a reference framework for assay design. The present study aimed to establish a standardized skin sensitization test method based on the domestically developed KeraSkin™ RhE and to develop a standard operating procedure (SOP) to support its practical application in the biohealth industry. Experimental conditions were established, including the selection of positive control concentrations, test chemical dose, exposure time, and RNA extraction procedures. Two positive control chemicals, 4-nitrobenzyl bromide and clotrimazole, were used to determine appropriate treatment volume and exposure duration. The optimization process enabled evaluation of the applicability of KeraSkin™ RhE and identification of experimental parameters that maintain cell viability while ensuring reproducible marker gene expression responses. The established method demonstrates the feasibility of applying KeraSkin™ RhE to keratinocyte activation-based skin sensitization testing and is expected to facilitate broader regulatory application and international recognition of domestically developed alternative test methods. Keywords: Skin sensitization, KeraSkin™ RhE, KoCVAM Funding Source: This research was supported by a grant (25211MFDS001) from the Ministry of Food and Drug Safety.

P130
A reconstructed canine epidermis model as an alternative approach for local tolerance assessment in veterinary pharmacology and pet care
ABSTRACT #343

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Veterinary pharmacology is a rapidly expanding field encompassing dermatological treatments, biotherapies, vaccines, and other locally or transdermally administered products intended for animal health. Ensuring local tolerance and safety of these products remains challenging due to species‐specific skin physiology, behavioral factors influencing exposure, and the limited relevance of human‐based models. At the same time, there is increasing scientific and ethical pressure to replace or reduce animal testing through the development of robust non‐animal methodologies. This work presents the development and intended applications of an in vitro reconstructed canine epidermis (RCE) model designed to support local toxicity and tolerance assessment for a wide range of veterinary and pet care products. The model reproduces key architectural and functional characteristics of canine epidermis and is compatible with established in vitro safety endpoints commonly used to assess skin integrity, cellular viability, inflammatory signalling, and tissue morphology. By combining multiple complementary biological parameters, the model enables a mechanistic and integrated evaluation of local effects following controlled exposure. The RCE model is positioned as a flexible decision‐support tool within tolerance and safety assessment strategies. It can be applied to ingredient screening, formulation optimization, and comparative assessment of candidates during early development, thereby supporting rational dose selection and reducing reliance on in vivo studies. Importantly, the model is adaptable to diverse exposure scenarios relevant to veterinary pharmacology, including leave‐on, rinse‐off, occlusive, and repeated‐contact conditions, as well as products with dermal or transdermal routes of administration. Beyond pet care and hygiene products, this reconstructed canine epidermis model offers significant potential for the non‐animal evaluation of veterinary medicines and vaccines requiring dermal safety assessment. Overall, it represents a promising advance in non‐animal local toxicity testing, supporting innovation in veterinary pharmacology while aligning with One Health principles and the 3Rs framework.

P131
Tissue-specific effect of antiparasitic substances. An in vitro approach. ABSTRACT #369

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Although antimicrobials are designed to act against pathogenic microorganisms, they can affect non-target organisms. Attention has been paid to the identification of antibiotics in the environment and their effects; however, scarce is the information related to the potential effects of other antimicrobials, as antiparasitics, on the ecosystems. Our aim was to evaluate the possible effect in fish of widely used antiparasitics. In vitro assays in two cell lines derived from different tissues of rainbow trout were conducted. Albendazole, ivermectin and praziquantel, commonly used as veterinary anthelmintics, were selected due to their wide distribution and lack of aquatic hazard data. Since those substances are achieving water sources, the selected cells were representative of both uptake vias, water contact (RTgill-W1) and ingestion (RTL-W1). Cells were exposed to a range of concentrations (0.78-100 µg/mL; ½ dilution) for 24h. The strong effect detected for albendazole led to test a wider range (0.003-100 µg/mL). Cytotoxicity was evaluated at the mitochondrial activity, plasma membrane or lysosomal functionality levels. Different effects were found depending on the cell line. The three antiparasitics were toxic for RTgill-W1 with IC50 of 13.46±0.77 µg/mL, and 69.56±6.05 µg/mL for ivermectin,and praziquantel, respectively. Albendazole was toxic at concentrations as low as 0.003 µg/mL. The toxicity in RTLW1 was similar for ivermectin (IC50 of 18.5±0.72 µg/mL), whereas praziquantel was not toxic up to 100 µg/mL and the LOEC for albendazole was 0.19 µg/mL. Albendazole induced its highest toxicity at the mitochondrial level in both cell lines as well as praziquantel in the RTgill-W1. No specific intracellular toxicity was observed for ivermectin. In general, branchial cells were more sensitive to antiparasitics. These makes relevant to evaluate different cell lines, from

different tissues, before achieving conclusions facing the cytotoxicity of substances. Funding: Agreement (MM SUBV 01 2025) with Diputación de Cáceres (Spain) through the Consorcio MasMedio.

P132
Development of a 3D atopic dermatitis model using keraskin™ and its application in preclinical efficacy evaluation
ABSTRACT #382

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Atopic dermatitis (AD) is a chronic skin disease characterized by barrier dysfunction and Th2-mediated inflammatory responses. Traditional animal models, however, have been criticized for their inability to fully recapitulate human pathophysiology, creating a growing need for human-relevant in vitro models. Consequently, the use of such models in preclinical evaluation has become increasingly important. There is also a recent emphasis on non-animal alternatives (New Approach Methodologies, NAMs), with regulatory authorities such as the FDA allowing the inclusion of human cell-based data in preclinical reviews. In this study, we developed a 3D Atopic Dermatitis (AD) model using the KeraSkin™ reconstructed human epidermal platform. Key pathological features of atopic dermatitis were induced by cytokine stimulation. Barrier function was assessed by TEER, while inflammatory responses and barrier protein expression (IL-6, IL-8, TSLP, Loricrin, Filaggrin, and Involucrin) were analyzed. Comparison with a normal skin model confirmed that key pathological features of AD, including decreased barrier function, increased Th-related inflammatory factors, and altered barrier protein expression, were recapitulated. In addition, referencing negative (NC) and positive (PC) control data demonstrated the model's potential as a reliable efficacy evaluation platform. This 3D Atopic Dermatitis (AD) model can serve as a human preclinical platform for screening cosmetic and related pharmaceutical candidates, and for conducting reliable efficacy evaluations regardless of formulation type. Key words: Atopic Dermatitis, Reconstructed Human Epidermis, KeraSkin™, Inflammatory Cytokines, Efficacy Evaluation, 3D model

P133
Toxicological evaluation of pristine MXene, polypyrrole-functionalized MXene, and MXene–graphene hybrids using cellular and computational models
ABSTRACT #386

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The SAFARI project aims to develop safe and sustainable formulations of MXene ($Ti_3C_2T_x$), MXene-polypyrrole ($Ti_3C_2T_x$-PPy), and MXene-graphene oxide ($Ti_3C_2T_x$-GO) for applications in electronics, energy storage, and biomedicine [1]. However, the increasing production of these nanomaterials raises concerns regarding potential human exposure through inhalation, dermal contact, and ingestion, while toxicological data remain scarce [2]. Integrated New Approach Methodologies (NAMs) are therefore urgently needed to support Safe-and-Sustainable-by-Design (SSbD) strategies and occupational risk assessment. To address this challenge, we implemented a three-tiered strategy. First, an initial hazard screening (Step 1-SSbD) was performed using QSAR-based predictive models to evaluate intrinsic material properties. Second, experimental safety assessment was conducted in human lung (A549) and liver (HepG2) models using conventional 2D monocultures, 3D spheroids, and dynamic 3D liver spheroids (3DD-HepG2) that better recapitulate tissue architecture and nanoparticle-cell interactions. Third, in silico modeling was employed to predict particle accumulation and protein-membrane interactions, providing mechanistic insight into nano-biointerfaces. QSAR analysis suggested that most MXene-based materials could not be

classified as inherently safe and therefore require precautionary handling. In vitro dose-response studies (0.78–500 µg/mL, 24 h) revealed limited cytotoxicity in 2D cultures (<100 µg/mL) [3], whereas 3D and dynamic spheroid models exhibited concentration-dependent responses and shifts in IC_{50} values, highlighting their increased physiological relevance and sensitivity. Overall, the integration of computational modeling with advanced in vitro systems provides a robust NAM-based framework for MXene hazard characterization. This approach addresses current gaps in nanomaterial categorization, supports SSbD development, and contributes to occupational risk mitigation in alignment with the 3Rs principles.

P134
Comparative hepatotoxicity assessment of a magnetic colloidal suspension containing oleic acid-double functionalized iron oxide nanoparticles in 2D and 3D liver models
ABSTRACT #424

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Magnetic iron oxide nanoparticles (MIONPs) exemplify one of the most studied classes of materials in biomedicine, distinguished by unique physicochemical properties, low toxicity, innate therapeutic activity, and various applications such as targeted drug delivery, hyperthermia, tissue repair, and imaging. To further ensure their proper stability for biomedical use, the field has gradually progressed toward functionalized MIONPs, often achieved through surface modification with coatings such as oleic acid (OA). Although the safety of MIONPs has been widely explored previously, few studies have researched the potential toxicity of MIONPs following OA-coating. Given that the liver serves as a primary site ensuring the capture and biodistribution of MIONPs, it was of interest for this study to investigate the in vitro hepatotoxicity of a colloidal suspension containing double-layer OA-coated MIONPs (sample MCS_OA) obtained through combustion for potential therapeutic applications. This aim was achieved following a 24 h MCS_OA treatment of HepG2 cells cultured as monolayers and spheroids, which had previously served as experimental models in nanotoxicity screenings. The findings revealed a concentration-dependent intracellular accumulation and cytotoxicity in HepG2 cells, outlined by a gradual reduction in cell viability and confluence, altered morphology, nuclear fragmentation, and constriction of F-actin and tubulin filaments. Notably, this behavior was maintained in HepG2 spheroids, with observed loss of viability, reduced size, and structure disorganization following treatment. Nonetheless, MCS_OA was found to induce hepatotoxicity in vitro only at high concentrations, while low MCS_OA doses lacked cytotoxic potential in these liver models.
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P135
Skin corrosion/irritation: evaluating the readiness of NAMs for regulatory decision-making
ABSTRACT #433

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New approach methodologies (NAMs) for skin irritation and corrosion (e.g. OECD TG 431 and 439) are internationally validated, yet their performance versus the most well known in vivo study (OECD TG 404) in real-world regulatory dossiers remains a key question for experts and regulators. More recently, EPA issued a

decision framework from the new chemicals program on skin irritation and corrosion (1) and has been advocating for the use and implementation of Defined Approaches (DAs) in their registrations (2). The objective of this work was to assess the comparative classification performance (concordance and relative conservatism) of in vitro assays relative to in vivo skin irritation/corrosion study outcomes, using both UN GHS/CLP and EPA's evolving NAMs strategy and decision frameworks for irritation/corrosion as reference frameworks for in vivo acute toxicity waivers. The analysis identifies conditions under which in vitro data alone would have supported equivalent regulatory decisions as in vivo testing, as well as situations where additional weight of evidence or integrated approaches (IATA) remain necessary. By benchmarking our internal dataset, this work will inform future data generation strategies under the EU Plant Protection Products (PPP) Regulation (EC) No. 1107/2009 and comparable global PPP frameworks, helping to meet skin irritation/corrosion data requirements while advancing worldwide implementation of the 3Rs policy (replacement, reduction and refinement). Keyworks: Skin; NAMs; DAs and irritation/corrosion.

P312
Vaginal irritation testing: towards regulatory acceptance of a method using reconstructed human vaginal epithelium
ABSTRACT #99

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Personal lubricants may or may not be classified as medical devices, depending on the country, product description and claims made. When classified as medical devices, manufacturers may be required among others tests to perform a rabbit vaginal irritation (RVI) test, in which live animals receive repeated applications of the product under evaluation before being killed. Despite growing support for non-animal methods, regulatory uptake remains limited. Non-governmental organizations (NGOs), such as the PETA Science Consortium International e.V., play a central role in aligning efforts among manufacturers, test developers, standardization bodies, and regulatory agencies to advance regulatory acceptance of human-relevant alternatives. The EpiVaginal test method is based on reconstructed human vaginal epithelium and may offer a scientifically robust alternative to the RVI test. Scientific confidence in the EpiVaginal test method is evaluated based on a well-established framework that considers the method's context of use, human biological relevance, technical characterization, data integrity and transparency, and independent review. Proof-of-concept studies demonstrate the test method's ability to rank water-based personal lubricants by their irritation potential. This presentation will detail the EpiVaginal test method qualification plan and discuss regulatory and standardization challenges to international acceptance.

Models, biomarkers and assays for developmental toxicity

P136
Advancing developmental toxicity testing with species-specific in vitro methods
ABSTRACT #134

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Developmental toxicity testing traditionally relies on animal models (primarily rats and rabbits) and is one of the most animal-intensive testing endpoints according to the current regulatory guidelines. Thus, developing new approach methodologies for developmental toxicity testing is essential to reduce the use of animals. However, few in vitro methods exist for predicting developmental toxicity in rats or rabbits, highlighting the need for species-specific in vitro approaches. We aimed to develop the RatDevTox assay, following the principle of a human induced pluripotent stem cell-based assay, ReproTracker: differentiation

of cells towards three lineage-specific cell types (cardiomyocytes, hepatocytes, and neuronal cells). So far, we have successfully differentiated rat embryonic stem cells into cardiomyocytes. Cardiomyocyte differentiation was investigated by morphological profiling and assessing time-dependent expression of cell-specific biomarkers. Any alteration in the expression of biomarker genes and morphology of differentiated cells following compound exposure indicates teratogenicity. To validate the assay, three well-known teratogens (retinoic acid, thalidomide, and tacrolimus) and two non-teratogens (saccharin and saxagliptin) were tested. Exposure to non-cytotoxic concentrations of retinoic acid disrupted cardiomyocyte differentiation by downregulating Flk1 (mesodermal-biomarker) and Myh6 (cardiac-biomarker), and inhibiting cardiomyocyte contraction. Exposure to thalidomide reduced the expression of Flk1 and Myh6 only at the highest tested concentration (100μM), without affecting beating. This result is consistent with thalidomide's in vivo teratogenicity classification, teratogenic to humans but less clear to rats. Exposure to saccharin and saxagliptin induced no significant effects on cardiomyocyte differentiation. The current study demonstrates that the RatDevTox assay is a valuable tool for predicting developmental toxicity in rats. Providing such a species-specific approach could advance the future of chemical safety assessment with fewer animal studies, aligning with the 3Rs principles. Further validation with a broader range of compounds will strengthen the assay's application in regulatory toxicology and advance the development of in vitro models for other species.

P137
Developmental neurotoxicity of dibutyl phthalate and its primary metabolite mono-n-butyl phthalate in zebrafish embryo model
ABSTRACT #187

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Mono-n-butyl phthalate (MBP), a major active metabolite of the plasticizer dibutyl phthalate (DBP), is a widespread environmental contaminant. While the toxicity of DBP has been extensively investigated, the potential hazards of its metabolite MBP remain insufficiently characterized. This study aimed to elucidate and compare the neurotoxic effects of DBP and MBP in zebrafish (Danio rerio) larvae. Wild-type and transgenic lines—tg(huC:eGFP) and tg(mbp:mGFP)—were exposed to DBP or MBP during early developmental stages (4–120 hours post-fertilization). Behavioral endpoints, including spontaneous tail coiling, touch-evoked response, and locomotor activity (both individual and group), were evaluated. Neurodevelopmental alterations were assessed through fluorescence imaging of transgenic lines and the expression of eight neurodevelopment-related genes. In addition, neurotransmitter levels and oxidative stress markers were analyzed to explore underlying mechanisms. Exposure to MBP and DBP resulted in reduced neurogenesis (tg(huC:eGFP)) and myelination (tg(mbp:mGFP)), accompanied by behavioral impairments such as decreased touch responsiveness and locomotion. MBP exposure further led to diminished dopamine levels and elevated oxidative stress. Both compounds exhibited distinct neurotoxic profiles, with MBP showing comparable or, in certain endpoints, even greater toxicity than DBP. These findings highlight the importance of considering metabolite-mediated toxicity. This work was supported by Korea Environment Industry & Technology Institute (KEITI) through Technology Development Project for Safety Management of Household Chemical Products Program, funded by Korea Ministry of Environment (MOE) (2022002980005, 1485019201) and the Korea Institute of Toxicology (KIT) Research Program (no. 2710008763, KK-2501-01).

P138
From DNT to DART: leveraging a NAM success story
ABSTRACT #195

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Background. Regulatory hazard and risk assessment of chemicals is shifting from animal

apical endpoints to human-relevant new approach methodologies (NAMs). Traditional animal studies are time- and resource-intensive, ethically problematic, and limited by interspecies differences. A domain of particularly high animal use and pronounced species specificity is developmental and reproductive toxicity (DART). Although exposures to certain chemicals have been associated with infertility, miscarriage, and birth defects, human-relevant test systems are limited. Method. The International STakeholder NETwork (ISTNET)-DART aims to build consensus on the use of in silico and in vitro methods to define a NAM-based integrated testing strategy that meets regulatory requirements for DART. The approach follows the successful ISTNET initiative for developmental neurotoxicity (DNT), where early involvement and dialogue among regulators, industry, and academia culminated in OECD Guidance Document No. 377. The first ISTNET-DART meeting (12–13 September 2024) followed this strategy and was intended to develop a roadmap for NAM-based DART testing involving all stakeholders. Result. The meeting brought together 61 participants from 12 countries across regulatory, academic, and industry sectors. 18 short talks covered reproductive and developmental biology, the status of in vitro methods, and physiology-based kinetic modelling. The talks were framed by regulator perspectives on current DART strategies, their perspectives on the use of NAMs in this complex area, their integration into next generation risk assessment frameworks, and views on readiness and validation. Consensus points included the challenge to cover all DART life-stages, the need for DART in-vitro batteries and tiered strategies, limited AOP coverage, and the value of in silico tools. Next steps are to disseminate outcomes as a DART roadmap to engage more scientists and regulators. Broader implications. With early stakeholder alignment and a biology-first approach, ISTNET-DART aims to support efficient tiered DART testing that delivers comprehensive, regulatory-relevant human health assessment.

P139
Human stem cell-derived osteoblasts for in vitro assessment of skeletal malformation

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Developmental toxicants are known to induce adverse outcomes during embryonic development, leading to growth retardations and malformations. Embryofetal developmental (EFD) studies in rat and rabbits remain the golden standard to identify adverse outcomes such as skeletal malformations. Human induced pluripotent stem cells (hiPSC)-based new approach methods (NAMs) offer a cost- and time efficient in vitro alternative that aligns with the 3R principles used for developmental and reproductive toxicity (DART) testing. The ReproTracker assay offers a platform to determine the teratogenic potential of chemicals based on the disruption of the development of hiPSCs into neural rosettes, hepatocytes and cardiomyocytes. In this study, the biological coverage of the ReproTracker assay was expanded by introducing osteogenesis to the platform. Differentiation of hiPSCs into osteoblasts was assessed through the analysis of both gene expression and protein levels of the mesodermal markers BRACHYURY and FLK1 and the bone-specific markers RUNX2 and COL1A1. Effects of well characterized in vivo bone developmental toxicants and non-developmental toxicants were assessed at non-cytotoxic concentrations. The teratogenic agents tested affected the expression of either mesoderm marker BRACHYURY at day 4, and/or bone biomarkers RUNX2, COL1A1 at days 7, and 14. Non-teratogenic agents did not affect the expression of bone biomarkers, showing the potential of these key biomarkers and how they can be used to identify bone specific teratogens. Integration of osteogenesis extends the biological coverage of the assay. Moreover, bone specific biomarkers can provide information of the mode of action of skeletal developmental toxicants, offering extra insights into skeletal end points. Overall, this new development enhances the assay's predictive capacity for identifying skeletal developmental toxicants.

P140

Qualification and quantification of a novel human in vitro syncytiotrophoblastic barrier: A predictive tool for maternal exposure. ABSTRACT #288

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Prenatal development, a critical phase of the reproductive cycle, is characterized by its immense complexity, driven by a formidable diversity of underlying biological mechanisms. Furthermore, parameters such as the developmental stage or external exposure sources can lead to equally diverse consequences. Considering the current regulatory context and the cosmetic industry's commitment to its values in developing innovative New Approach Methodologies (NAMs), it appears necessary to design an in vitro human placental barrier model that addresses both scientific and industrial challenges. The development of such in vitro models could help us understand, mimic, and predict the functioning of this guarantor of fetal development, which is the site of numerous perfectly controlled exchanges. This co-culture model, representative of syncytiotrophoblastic and endothelial tissues, has been characterized at several levels: • Biological: A panel of 148 transporters and metabolic enzymes, described as essential for the proper functioning of this tissue, was quantified at both the transcriptomic and proteomic levels. • Functional: The model was exposed to a set of reference molecules, documented for having different passage mechanisms. The passage was measured by the analytical quantification of these molecules. These results support that the model exhibits a high level of predictivity, positioning it as a method of interest for objectively demonstrating the absence of molecular passage across the placental barrier. Ultimately, this model could become a valuable source of data for refining exposure models (PBK), relevant to the maternal population.

P141

Integrating hPSC-derived monolayer and brain-on-chip models as new approach methodologies for developmental neurotoxicity testing. ABSTRACT #344

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Traditional animal models often fail to predict human-specific developmental neurotoxicity testing (DNT) due to interspecies differences. Human-relevant New Approach Methodologies (NAMs) are needed to overcome the limitations of these models in predicting neurodevelopmental toxicology (DNT). We established two complementary hPSC-based models to evaluate neurodevelopmental processes and chemical safety. The first platform consists of a monolayer system optimized for high-throughput screening (HTS). Using our robust differentiation protocol, we generated hPSC-derived neural epithelial progenitors (hNEPs), neural progenitor cells (hNPCs), and mature ventral forebrain neurons. The monolayer platform was validated using a library of reference neurotoxicants (heavy metals, pesticides and drugs), demonstrating its suitability for high-throughput DNT screening and the identification of stage-specific susceptibility. Our results demonstrated dose-dependent toxicological responses, allowing us to determine the IC50 for each compound across different developmental windows. To address the functional complexity of the human brain, we developed a brain-on-chip model. This device allows the independent differentiation of hPSCs into region-specific neurons, enabling them to establish synaptic connections that recapitulate the ones in the basal ganglia circuitry. Functional analysis demonstrated that cortical

innervation significantly promotes the maturation of striatal neurons. Furthermore, the brain-on-chip model enables the assessment of chemical-induced disruptions in neuronal connectivity and network functionality, providing a more complex read-out for functional neurotoxicity. In conclusion, these models offer a robust and human-relevant approach to DNT, combining HTS with intricate functional assays. Furthermore, as they are NAMs, there is the possibility of alignment with the principles of the 3Rs, with a reduction in reliance on animal testing for chemical risk assessment and drug screening.

P142
Development of an in vitro placental barrier model to assess the teratogenicity potential of chemical compounds.
ABSTRACT #361

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Background and Objectives: Teratogenicity is one of the toxicological endpoints assessed during the safety assessment of pharmaceutical, cosmetic, and general chemical ingredients. Cosmetic ingredients classified as reproductive toxins are prohibited for use in products according to European regulations. Currently, several ingredients in the cosmetic industry, particularly fragrance ingredients, are undergoing hazard classification in Europe. The validated methods currently used to assess this hazard rely on animal testing. Therefore, the development of alternative methodologies to assess teratogenicity is crucial, as it remains a toxicological endpoint with no validated in vitro test. The existence of an in vitro methodology will help anticipate potential classifications, assisting in decision-making regarding ingredient bans, and product reformulations. To evaluate the teratogenic potential of substances, it is essential to understand their ability to cross the placental barrier and reach the embryo/fetus. Thus, the placenta is of critical importance in the assessment of this toxicological endpoint. Material and Methods: This study aimed to develop an in vitro placental barrier equivalent using cell lines JEG-3 and HUVEC in an insert. The characterization of this organ equivalent was performed using cell viability assays as MTT and Live/Dead, Transepithelial/Transendothelial Electrical Resistance (TEER) measurement, gene expression, permeability and imaging techniques. Results: Characterization data shows a robust barrier equivalent, with TEER measurement consistent with data from the literature. Furthermore, favorable viability results were obtained, showing that the tissue cells were viable days after biofabrication. Furthermore, gene expression confirmed the presence of markers associated with placental physiology, biological mechanisms, and embryonic development. Discussion and Conclusion: Preliminary results indicate a promising placental barrier model for in vitro teratogenicity studies. Further characterization techniques will be applied to complete the validation of the model and integration into a microphysiological system alongside other organ equivalents to assess the teratogenic potential of substances of interest.

P143
Metabolomics and lipidomics for neurotoxicity assessment in human 3D brain spheres: an example with glycol ether solvents
ABSTRACT #367

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Glycol ethers are widely used organic solvents, found in paints, inks, cleaning products and cosmetics, among others. Exposure to these solvents are associated with cognitive deficits, however their neurotoxic effects have not yet been systematically evaluated. Ethylene-based glycol ethers (EGEs) are considered to exhert their toxic action through their main metabolites, the alkoxyacetic acids. Consequently, they are being replaced with propylene glycol ethers (PGEs), containing a mixture of primary and secondary alcohols. While the secondary alcohols are metabolised to non-toxic products, primary alcohols of PGEs produce alkoxypropionic acids, which could be neurotoxic. PGEs have indeed been reported to cause headaches via vapor inhalation in humans, and in rat 3D cell culture propylene glycol butyl ether (PGBE) was found to be more neurotoxic than the banned EGME. Recently, the use of -omics approaches on three-dimensional cell cultures has gained attention for toxicity assessment at the molecular level. In this study, we aim to investigate metabolomic and lipidomic shifts associated with neurotoxicity in PGBE and its main metabolite, 2-butoxypropanoic acid (2-BPA). Human induced pluripotent stem cells were differentiated for 7 weeks into neurons and glial cells (oligodendrocytes and astrocytes) in 3D spheroids, mimicking the brain microenvironment in vitro. Brain spheres were then exposed to 5, 10 and 20 mM PGBE and its metabolite 2-BPA for 48 h and 1 week. Polar metabolomics led to 120 level 1 annotations. OPLS-DA data analysis showed altered levels of ribonucleotides, suggesting an impact of glycol ethers exposure on the brain energy metabolism. Untargeted ipidomic analysis, based on MS^2 annotation of 713 lipid species, revealed significant decreases of phosphatidylethanolamines and hexosylceramides after solvents exposure. These findings, complemented by other omics analyses, contribute to a deeper understanding of PGE-induced neurotoxicity.

P144
A regulatory-relevant human placental model to identify early endocrine and molecular disruptions
ABSTRACT #383

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Background The placenta is a multifunctional organ that supports fetal development through barrier, immune and endocrine functions, and its disruption by drugs or chemicals can lead to adverse pregnancy outcomes. We developed a human placental co-culture model that integrates functional, inflammatory, and molecular readouts to enable mechanistic evaluation of potential placental toxicants. Using this system, we assessed the developmental toxicity of bleomycin, a chemotherapeutic with limited and conflicting developmental toxicity data and low placental transfer. Methods A placental co-culture model comprising cytotrophoblasts (BeWo-cells), syncytialized trophoblasts (forskolin-treated BeWo-cells) and human vascular endothelial cells (HPVECs) on porous inserts was established. Endocrine and inflammatory functions were measured by ELISA (hCG and IL-6), and acute cytotoxicity by MTT and LDH assay, using IL-1β and $CdSO_4$ as positive controls. To investigate bleomycin-induced toxicity mechanisms, transcriptomic profiling was performed to identify molecular changes linked to placental stress and endocrine disruption. Results The co-culture model exhibited stable baseline endocrine activity. IL-1β induced robust IL-6 release, confirming functional inflammatory responsiveness, while $CdSO_4$ reduced cell viability, validating acute toxicity detection. Following 24h of bleomycin exposure, no mapped AOPs or canonical endocrine-related key events were detected. IL-6 levels remained unchanged, and no acute cytotoxicity was observed. Notably, hCG expression was significantly increased at both the transcriptomic level (CGB3 and CGB5) and the protein level (ELISA), indicating enhanced endocrine activity even in the absence of mapped key events. Conclusion Together, these findings show that our human placental co-culture model detects early functional and molecular perturbations, including hCG induction that can reflect placental stress linked to adverse pregnancy outcomes, while avoiding activation of canonical AOP pathways or acute toxicity. This demonstrates the model's value

as a mechanistic and regulatory relevant NAM platform for identifying subtle chemical disruptions of placental function and improving early hazard assessment of potential placental toxicants such as bleomycin.

P145
Non-exhaust particles from electric vehicles: evaluation in human skin and lung models within the LIFE-NEEVE project.
ABSTRACT #395

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Background and Objectives The global transition to electric mobility is widely regarded as a zero-emission solution; however, non-exhaust emissions (NEE) remain a critical and insufficiently characterized source of pollution. Tires, brake pads, and road pavements release particulate matter containing transition metals (e.g., Cu, Fe, Zn), carbonaceous materials, and polymer residues. The high surface reactivity of these particles raises concerns about their ability to induce oxidative stress, systemic inflammation, and epithelial barrier disruption upon human exposure. The LIFE-NEEVE project aims to address this challenge by developing and testing innovative solutions, including low-emission brake pads and optimized pavements, while establishing robust strategies for the collection and physicochemical characterization of NEE particles to bridge technical performance and human health impact. Material and Methods Particles from distinct sources were isolated and their physicochemical profiles correlated with biological responses using advanced 3D in vitro models. These New Approach Methodologies (NAMs) overcome limitations of conventional monocultures and align with the 3Rs principle to reduce animal testing. For dermal exposure, fibroblast–keratinocyte spheroids and a trilaminar organotypic skin model integrating epidermis, dermis, and a functional hypodermis were developed on PET scaffolds within collagen–fibronectin matrices, achieving maturation after five weeks at the air–liquid interface (1). For inhalation exposure, long-term expandable human airway organoids and pulmonary spheroids derived from basal cells replicated key structural and functional features of the respiratory epithelium (2, 3). Results Following validation of tissue viability, models were exposed to NEE particles to assess cytotoxicity, barrier integrity (TEER), inflammatory signaling, redox imbalance, and lineage-specific marker alterations. Discussion and Conclusion We hypothesize that particle composition and size distribution drive biological responses, with metal-rich and ultrafine fractions showing the highest pro-oxidant and pro-inflammatory potential. By integrating advanced material characterization with physiologically relevant human 3D models, LIFE-NEEVE provides mechanistic evidence to support scientifically informed strategies for sustainable and health-conscious mobility.

P146
Cross-species human and primate iPSC-based NAMs for in vitro developmental toxicity assessment of targeted protein degraders
ABSTRACT #398

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Background and Objectives: Targeted Protein Degraders (TPDs), including Proteolysis Targeting Chimeras (PROTACs), represent a promising drug class capable of targeting otherwise “undruggable” proteins in cancer and other diseases. Despite their promise, developmental and off-target toxicity remain key challenges, particularly for critical developmental transcription factors like SALL4, whose degradation is linked to teratogenic

outcomes observed with thalidomide analogs. Due to their close developmental and physiological similarity to humans, nonhuman primates (NHPs) are frequently used to evaluate these risks, yet in vivo NHP developmental studies pose substantial ethical, logistical, and financial challenges. To address this gap, we developed a cross-species New Approach Methodology (NAM) using human and cynomolgus macaque (cyno) pluripotent stem cell (PSC) model to assess TPD-induced developmental toxicity in vitro. Materials and Methods: Both PSC models were validated for endogenous SALL4 expression and engineered via AAV-mediated tagging of the SALL4 3′ exon to generate SALL4-TdTomato reporter lines, enabling non-destructive, longitudinal monitoring of protein degradation. Results: Cells were exposed to established degraders (thalidomide, lenalidomide, and pomalidomide) across a range of concentrations. Dose-dependent SALL4 degradation kinetics and EC_{50} values were quantified using live-cell fluorescence imaging and orthogonally validated by Data-Independent Acquisition (DIA) proteomics. SALL4 expression and degradation profiles were comparable between human and cynomolgus PSCs, with overlapping proteomic signatures supporting strong translational relevance. Notably, degradation efficiency was influenced by cell density, highlighting the advantage of dynamic reporter-based assays over static endpoint measurements. Discussion and Conclusion: This study establishes a scalable, ethically sustainable, human–cynomolgus iPSC NAM platform for evaluating developmental toxicity of protein degraders. By integrating live-cell reporters with global proteomics, this approach provides a translational bridge between in vitro safety assessment and NHP in vivo findings, supports 3Rs principle (Replacement, Reduction, Refinement), and enables informed ranking of TPD candidates prior to animal testing.

P147
Evaluation of neurodevelopmental disruptions induced by emerging Bisphenol analogues using zebrafish embryo
ABSTRACT #409

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Bisphenol A (BPA), a widely used endocrine disruptor in plastics, has been restricted due to proven health hazards. First-generation alternatives (BPS, BPF) showed similar toxicity, prompting regulations. Second-generation substitutes (BPE, BPG, BPP, BPZ) are now extensively used without adequate hazard assessment, particularly for developmental neurotoxicity. This study systematically investigates the developmental neurotoxic effects of four second-generation BPA substitutes using zebrafish embryos as an alternative testing model. Zebrafish embryos were exposed to environmentally relevant concentrations of BPA analogues following OECD guidelines and evaluated through neurobehavioral assessments, transgenic imaging (neurogenesis and myelination), gene expression analysis, and blood-brain barrier integrity testing. BPA alternatives exhibited significant developmental neurotoxicity at concentrations 10-fold lower than BPA. BPE and BPP induced more pronounced neurotoxic phenotypes than BPA, showing behavioral disruptions including reduced swimming speed, increased anxiety-like responses, which revealed disrupted neural network function. Decreased neurogenesis and myelination reflected primary neuronal dysfunction, and neurodevelopmental genes were downregulated. BPP particularly exhibited gene expression patterns closely resembling BPA, suggesting similar neurotoxic mechanisms. Findings were supported by altered acetylcholinesterase activity and increased blood-brain barrier leakage. These results demonstrate that certain BPA analogues cause adverse neurodevelopmental effects at doses below those associated with BPA, highlighting the urgent need for comprehensive toxicological profiling before widespread adoption. This work was supported by Korea Environment Industry & Technology Institute (KEITI) through Technology Development Project for Safety Management of Household Chemical Products Program, funded by Korea Ministry of Environment (MOE) (RS-2022-KE002135).

P148

Development of regulatory toxicity indicators for biocides: bridging transgenic zebrafish imaging with AOP-based human health risk assessment

ABSTRACT #411

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To address neurotoxicity concerns and comply with South Korean biocidal regulations, this study evaluated 50 frequently used biocidal substances using transgenic zebrafish models. We determined LC25 values via OECD TG 236 to standardize exposure concentrations for behavioral and developmental assays. Neurotoxicity was assessed through behavioral tests including tail coiling, touch-evoked response, and locomotor activity and morphological analysis of CNS development and myelination using three transgenic lines. Exposure to specific biocides led to significantly impaired CNS development and reduced neuronal myelination, characterized by decreased fluorescence intensity compared to controls. Using the Comparative Toxicogenomics Database (CTD), we identified strong correlations between these biocides and genes associated with human neurological disorders, such as multiple sclerosis. By integrating in vivo experimental data with the Adverse Outcome Pathway framework and existing toxicological databases, this study provides a comprehensive approach to identifying risks to aquatic ecosystems and human health. These findings support the development of robust regulatory toxicity indicators for biocidal products.

P149
High-throughput quantitative analysis of PLP1-positive oligodendrocytes in a 3D human iPSC-derived brain model for chemical screening

ABSTRACT #428

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Neurodevelopmental disorders affect over 15% of the global population, with environmental toxicants contributing significantly to their prevalence. Current regulatory developmental neurotoxicity (DNT) assessments are costly, resource-intensive, and require large animal numbers. Consequently, these tests are rarely conducted routinely, leaving many chemicals without proper evaluation. In response, the Organisation for Economic Co-operation and Development (OECD) and the European Food Safety Authority (EFSA) together with international experts developed a DNT in vitro battery covering key brain development events. However, assays addressing the oligodendrocyte (OL) lineage remains a critical gap, special for myelination. Because functional myelination involves extensive developmental timelines in vitro, our study focuses on the pre-myelinating OL stage as a highly sensitive and predictive window for chemical interference. We used a 3D human "BrainSphere" model derived from human induced pluripotent stem cells engineered with a proteolipid protein 1 (PLP1)-GFP reporter to identify the transition to pre-myelinating OLs. To enable high-throughput screening, we implemented an automated high-content imaging assay to evaluate the morphology and the structural integrity of PLP1+ cells across a chemical library. To complement imaging, flow cytometry was employed to quantify the PLP1+ OLs within the total population. Over 8 weeks, the model showed a consistent increase in PLP1+ signal. Flow cytometry detected significant shifts in the PLP1+ population following a 1-week exposure to a preliminary panel of compounds, identifying both enhancers and inhibitors of OL development in the absence of general cytotoxicity. High-content imaging further identified compounds that altered PLP1+ signal intensity and cellular morphology. In conclusion, integrating automated imaging with flow cytometry allows for the efficient quantitative screening of pre-myelinating OLs. By detecting stressors affecting these cells early in development, this human-relevant platform provides a powerful

tool for predicting later impairments in cellular maturation and neuronal support.

P150
The devTOX quickPredict™ assay: blinded study demonstrates high inter-laboratory reproducibility for in vitro developmental toxicity NAM
ABSTRACT #434

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Assessing a NAM's reproducibility is a necessary step to establish confidence in a method and enable its use in a regulatory setting. For Developmental and Reproductive Toxicity (DART) testing, some regulatory agencies have released guidelines that permit the use of NAMs in conjunction with or in place of in vivo embryo-fetal development (EFD) studies. The devTOX quickPredict (devTOXqP) assay is an in vitro human pluripotent stem (hPS) cell-based assay that predicts the developmental toxicity potential of chemicals with high accuracy based on changes in ornithine and cystine metabolism, represented as the ornithine:cystine ratio (o/c ratio). It has been used by multiple industries for compound prioritization for over a decade and is included as part of the Next Generation Risk Assessment (NGRA) strategies being developed by multiple companies for animal-free assessment of DART testing. The interlaboratory reproducibility of the devTOXqP assay was performed at Labcorp (following full training) by testing eight compounds with known developmental toxicity profiles (2 non-developmental toxicants, 6 developmental toxicants) previously tested by Stemina. The developmental toxicity potential (dTP, o/c ratio) and toxicity potential (TP, cell viability) concentrations were calculated from both laboratories and results compared to assess assay reproducibility. The Labcorp data yielded o/c ratio curves for the six developmental toxicants tested and no response was observed for the two non-developmental toxicants. These data were fully concordant with the results obtained at Stemina for the same compounds. The difference in the dTP and TP concentrations was less than three-fold between Labcorp and Stemina, demonstrating excellent assay reproducibility. Testing 6 developmental toxicants and 2 non-developmental toxicants (each with known developmental toxicity profiles) in two independent laboratories using a blinded study design has demonstrated that the devTOX quickPredict™ assay is a highly reproducible NAM and can be used with confidence for prediction of developmental toxicities in vivo.

P329
Neuroinvasive potential of microplastic-associated microbial biofilms: mechanisms, pathogenesis, and therapeutic interventions via integrated in vitro and in vivo modelling (zebrafish)
ABSTRACT #445

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Microplastics (MPs) have emerged as pervasive environmental contaminants with implications that extend beyond physical toxicity to complex biological interactions. Acting as vectors for diverse microbial communities, MPs provide a unique ecological niche that supports the attachment, survival, and transport of bacteria, fungi, and potentially pathogenic organisms. This microbial colonization enhances the capacity of MPs to function not only as pollutants but also as carriers of infectious agents, raising concerns about their role in human health. A critical and relatively underexplored aspect of MP exposure is their potential to facilitate the translocation of microbes across biological barriers, including the gastrointestinal epithelium and the blood–brain barrier (BBB). Once internalized, MPs may act as Trojan horses, enabling associated microbes or their toxins to reach the central nervous system (CNS). This process could contribute to neuroinflammation, disruption of neuronal homeostasis, and the onset of neuroinfectious or neurodegenerative conditions. Although experimental studies have demonstrated the

neurotoxic effects of MPs—such as oxidative stress, inflammation, and altered neurotransmission—the specific contribution of MP-associated microbial contamination to CNS invasion remains poorly understood. Furthermore, the interaction between MPs, host immune responses, and microbial virulence factors represents a complex triad that warrants detailed investigation. Understanding the molecular and cellular mechanisms underlying these interactions is essential for identifying potential therapeutic targets. Current research lacks comprehensive strategies to mitigate the combined toxicological and infectious risks posed by MPs. Therefore, there is an urgent need to develop targeted interventions, including antimicrobial coatings, biodegradation approaches, and pharmacological therapies, to prevent or reduce MP-mediated neurotoxicity. In conclusion, microplastics represent a multifaceted threat, bridging environmental pollution and infectious disease pathways. Elucidating the mechanisms of MP-associated microbial translocation into the CNS will be critical for advancing both preventive and therapeutic strategies in this emerging field.

P330
Zebrafish embryotoxicity test (ZET) for the screening of difficult-to-test chemicals for developmental toxicity
ABSTRACT #460

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Background and Objectives: Studies are needed to explore the potential human toxicity of Substances of Unknown or Variable Composition, Complex Reaction Products, or Biological Materials (UVCBs), as well as difficult-to-test substances, including those with low water solubility. The zebrafish embryo has been proposed as a new approach methodology (NAM) that enables the study of the embryotoxic potential of chemicals. Material and Methods: Eight difficult-to-test substances that are used in the lubricant industry, including multiple UVCBs and substances with low water solubility, were tested in zebrafish embryos by chemical incubation and microinjection. Initially, a dose-range finding assay was completed to determine the conditions and concentrations at which malformations and lethality were induced. A definitive experiment was subsequently conducted, and teratogenic index (TI) values were calculated based on the computation of lethality and embryotoxicity parameters (LC50, EC50, and NOAEL). Each of these substances had previously been tested in validated mammalian assays to assess reproductive or embryotoxicity. Results: The zebrafish assay correctly predicted all three test substances that were not embryotoxic in mammalian reproductive or developmental assays. The zebrafish assay correctly predicted three of the five remaining substances that were classified for developmental toxicity based on prior mammalian assays using the incubation method but not the microinjection method. The two substances that were not correctly predicted by the zebrafish embryo assay produced less severe effects in the mammalian developmental screening assays. Discussion and Conclusion: The use of the zebrafish developmental toxicity assay for screening UVCBs and difficult-to-test substances allowed the successful determination of their embryotoxic potential. The assay can help prioritize the screening of substances early in development or as part of a weight of evidence in regulatory submissions. The project also helped to expand the domain of applicability for this NAM.

Models, biomarkers and assays for endocrine disruption

P151
Development and validation of a sodium iodine symporter (NIS) inhibition assay using a hNIS overexpressing cell model
ABSTRACT #136

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Background and Objectives Proper thyroid hormone homeostasis plays a crucial role in human development and basal metabolic rate and disruption of thyroid hormone homeostasis may lead to severe neurodevelopmental and physical defects. The Sodium Iodine Symporter (NIS) facilitates the active transport of iodide into cells and therefore plays a critical role in

thyroid hormone synthesis and homeostasis. In addition, NIS is exploited in the diagnosis and treatment of thyroid cancer using e.g. radioiodine, but is also a potential target of xenobiotics. In this study, the development and in house validation of an in vitro NIS inhibition assay is reported Material and Methods A HEK293 cell line stably overexpressing human NIS was generated at Charles River SOLVO, Hungary. Using this cell line, initial experiments were conducted to determine optimal iodide uptake conditions (colorimetric Sandell-Kolthoff reaction) and cytotoxicity conditions (RealTime-GloTM). Next, the effect of 14 selected test chemicals on NIS-dependent iodide uptake was investigated and compared to the positive control for NIS inhibition, potassium perchlorate (KClO4). Test chemical selection was based on literature, earlier in vitro thyroid assay validations studies coordinated by EURL ECVAM and in-silico prediction. Results Ten out of 14 test chemicals did not inhibit iodide uptake via NIS. In addition to the positive control KClO4, four out of 14 test chemicals inhibited iodide uptake via NIS by >20%, including KPF6, NaBF4, NaSCN and strikingly also Tetraiodothyroacetic acid (TETRAC). Discussion and Conclusion The NIS assay was successfully developed and validated. Inhibition of iodide uptake by TETRAC was certainly not expected and further work is in progress to investigate if the observed inhibition is indirect or a direct effect on the NIS transporter itself and if the observed effect is TETRAC specific or a more general phenomenon for thyroid hormones / metabolites.

P152
Endocrine disrupting properties of water soluble metal working fluids: effects on steroid hormone production and receptor transcriptional activities.
ABSTRACT #144

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Background and Objectives: Metal working fluids have evolved considerably in recent decades, with changes in their composition aimed at making them water soluble and reducing the proportion of petroleum-based substances. Furthermore, the most controversial substances such as polycyclic aromatic hydrocarbons tend to be reduced or absent in these new formulations. However, because these fluids are now mostly aqueous or miscible in water, biocidal, foaming and anti-corrosive agents are often added. As a result, knowledge about the health hazard posed by these new cutting fluids is relatively limited. Material and Methods: In this study, we investigated the effects of selected aqueous cutting fluids samples on the production of testosterone and oestradiol by H295R cells, as well as their ability to modify the transcriptional activity of the androgen receptor in AT-EcoScreen CHO cells or the oestrogen alpha receptor in HeLa9903-hERα cells. Results: Our initial findings suggest that among the samples tested so far, some before-use cutting fluids have endocrine-disrupting effects in our experimental models such as the induction of oestradiol production in H295R cells or the transcriptional activation of its receptor in HeLa9903-hERα cells. Experiments comparing the toxicological properties of before-use fluid samples with their counterparts collected after use in machining parts, are ongoing. Discussion and Conclusion: To the best of our knowledge, this work is the first one showing such toxicological effects. However, at this stage, we have not been able to identify the molecule(s) present in the selected water-soluble metal working fluids responsible for their endocrine-disrupting properties.

P153
C. Elegans as an innovative biosensor for combined toxicity in surface water: exploratory insights from the INNOWACE project
ABSTRACT #158

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The increasing complexity of water pollution, characterized by mixtures of persistent substances such as PFAS, endocrine-disrupting chemicals (EDCs), pesticides, and microplastics, calls for innovative approaches to water quality monitoring. Within the framework of the INNOWACE project, we investigate the applicability of the nematode Caenorhabditis elegans (C. elegans) as a biosensor for detecting combined toxicity in surface water. Our preliminary data indicate that C. elegans is a sensitive and robust model organism for signaling toxic effects of both individual and combined contaminants. In controlled experiments with mixtures of PFAS, EDCs, and pesticides, we observed enhanced toxic effects on biological endpoints such as reproduction and development compared to exposure to single compounds. Remarkably, the addition of microplastics to these mixtures led to a significant decrease in observed toxicity, suggesting a possible sorption effect or interaction between microplastics and other pollutants. These findings highlight the importance of testing environmentally relevant mixtures rather than single substances and confirm the suitability of C. elegans as an alternative animal model for environmental toxicology research. Furthermore, the closed liquid culture system developed within this project provides a reliable and reproducible method for exposure to both hydrophilic and hydrophobic compounds. The INNOWACE project thus makes an important contribution to the development of a versatile, cost-efficient, and biologically relevant biosensor for water quality monitoring. The results form the foundation for a larger-scale follow-up project aimed at implementing C. elegans-based biosensors in the practical domains of water management and environmental monitoring.

P154
Towards serum-free, hormone-controlled culture conditions for in vitro steroidogenesis assays
ABSTRACT #174

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Serum-containing media introduce variability due to uncontrolled hormone levels, which can potentially interfere with endogenous steroid production in cultured cells, affect assay performance, and reduce reproducibility. This is particularly problematic for in vitro steroidogenesis assays, such as the H295R cell-based assay (OECD TG 456), where consistency and mechanistic relevance are essential for regulatory acceptance. Additionally, the use of animal-derived serum raises ethical concerns and conflicts with the 3Rs principle (Replacement, Reduction, Refinement). We aim to establish a serum-free, hormone-controlled culture protocol for key cell lines used in steroidogenesis assays, including H295R and Leydig TM3 cells. Our goal is to reduce variability, improve reproducibility, enhance human relevance, and decrease reliance on animal-derived products in vitro models assessing chemical impacts on reproductive health. Initial optimization focused on Leydig TM3 cells (immature, non-tumorigenic Leydig cells from 11–13-day-old mice), which were successfully adapted to serum-free conditions while maintaining viability and doubling time. We then applied hormone-controlled conditions to H295R cells and evaluated responses to physiologically relevant hormone levels, stimulants, and reference chemicals. Steroidogenic activity was assessed via enzyme expression and hormone production. Finally, we compared chemical effects under standard serum-containing conditions with those under the newly developed hormone-controlled protocol. Our findings support the transition toward more defined, ethically responsible, and mechanistically informative in vitro systems. This work contributes to the refinement of steroidogenesis assays and supports broader adoption of NAMs in regulatory toxicology.
Acknowledgment: This project is funded by the European Partnership for the Assessment of Risks from Chemicals (PARC), Grant Agreement No. 101057014.

P155
Refining predictive transcript markers for liver steatosis in metabolism-disrupting compound screening
ABSTRACT #184

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Metabolic disorders continue to rise globally, increasing the demand for advanced methods capable of accurately identifying metabolism-disrupting compounds (MDCs) and their effects on lipid and energy homeostasis. The present work builds upon in vitro data derived from differentiated HepaRG cells using the AdipoRed assay, demonstrating very good assay performance in quantifying triglyceride accumulation as a phenotypic indicator of steatosis. Subsequently, a transcriptomic marker set predictive for liver triglyceride accumulation was identified using LASSO regression, comprising the genes ANXA10, ARG1, CCL20, CYP2D6, INSIG1, POR, SLCO1B1, CD36, FASN, and SREBF1, of which the latter three represent key events within the AOP for steatosis (Lichtenstein et al., 2020). As previously described by Karaca et al. (2023), this marker set can be applied for screening both single compounds as well as their mixtures. The initial training set comprised 30 pesticidal active compounds. To evaluate the applicability domain of the transcript marker set, an extended test set of 46 additional substances from diverse chemical classes, including endocrine disruptors, mycotoxins, food contaminants/additives, food contact materials, and environmental pollutants was analyzed. Data were collected to assess predictivity. To enhance model robustness through machine learning-based optimization, the combined dataset of 76 compounds was used to retrain the model. The training set was redefined, and support vector machine and random forest algorithms were applied to refine gene selection for class discrimination, achieving an overall accuracy above 70 %. Furthermore, triglyceride accumulation was correlated with the transcriptomic marker set, identifying FASN, INSIG1, POR and SREBF1 as significant markers with similar predictive performance. Cross-validation (leave-one-out) confirmed the model's stability and minimized potential overfitting bias arising from pesticide overrepresentation in the original dataset. Overall, remodeling the combined dataset using machine learning enabled the refinement of predictive gene markers, resulting in more robust in vitro predictions of liver steatosis.

P156

NAM-based assay panel for evaluating compound effects on thyroid hormone synthesis
ABSTRACT #190

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Thyroid hormone (TH) signaling plays a central role in development, metabolism, neurocognitive function and the disruption of this axis can lead to adverse health outcomes. Current regulatory trends emphasize the need to identify the potential endocrine-disrupting properties of substances used in regulated contexts with increasing reliance on new approach methodologies (NAMs) to generate human-relevant and mechanistic evidence in place of animal testing. In a regulatory setting, NAMs which capture key molecular initiating events and downstream pathway effects represent valuable tools for early hazard identification and can also support weight of evidence evaluations in chemical safety assessments. In the case of However, determining whether a compound can interact with TH signaling requires more than assessing a single molecular event and multiple endpoints are needed to cover the different mechanisms involved in the pathway. Therefore, we assembled a panel of in vitro bioassays designed to evaluate compound-mediated effects at several key steps of TH synthesis. This integrated approach aims to capture mechanistic diversity of the thyroid endocrine system and enables differentiation of compound-specific modes of action. After their implementation, each assay underwent internal validation following general standards generally applied for in vitro assay validation and based on our experience with achieving regulatory acceptance (e.g., OECD TG 455, 458). Internal validation included testing a set of reference compounds along with additional compounds relevant to each assay. Once the assays met predefined acceptance criteria and demonstrated good performance, all test compounds were cross tested across the entire panel, revealing distinct activity profiles. The results highlight the value of integrated NAM-based strategies for assessing thyroid endocrine disruption. The assembled assay panel offers a practical, mechanistically informative tool for screening and comparative evaluation thus aligning with current regulatory trends toward non-animal testing and

supporting robust chemical safety assessments.

P157
Chlorpyrifos induces dimorphic effects in immortalized hypothalamic murine neurons in presence of physiological concentrations of sexual hormones
ABSTRACT #208

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Chlorpyrifos (CPF) is an organophosphorus pesticide still widely used in several countries. Human exposure mainly occurs through diet, with children and pregnant women being particularly vulnerable. CPF induces neurodevelopmental and reproductive adverse effects possibly by disrupting the hypothalamic-pituitary-gonadal (HPG) axis. Building on previous evidence of sexually dimorphic effects in hypothalamus of mice, we investigated the mode of action of CPF using the murine GT1-7 cell line of hypothalamic GnRH neurons, as main regulators of the HPG axis. Cells were treated with CPF (1 nM–100 µM) in absence or presence of 17β-estradiol (E2, 100 pM) or testosterone (T, 10 nM), assessing cell viability, proliferation, mitochondrial membrane potential (MMP), ROS production and spheroid morphology. At CPF non-cytotoxic doses (1–100 nM), we evaluated GnRH secretion by ELISA, and gene expression of GnRH, ERα, ERβ, OXT, CYP19A1, and OXTR by qPCR. TEM analysis was performed to evaluate cell morphology. Additionally, proteomic profiling is assessed. CPF reduced cell metabolic activity, especially with E2, whereas in presence of T proliferation was decreased. CPF strongly reduced MMP; the effect was partially rescued in presence of E2 and T. Accordingly, CPF alone increased ROS levels at the highest dose, but not in presence of hormones. TEM revealed protective effects of E2 and T against CPF-induced mitochondrial damage. Spheroid area grew at higher CPF doses with T. GnRH secretion decreased in cells treated with CPF+E2 at all concentrations and in CPF+T at the highest one, consistent with gene expression data. CPF+E2 upregulated ERα and OXTR, while CPF+T increased ERβ, CYP19A1, and OXT expression. Overall, CPF exposure of GT1-7 cells exerted dimorphic effects in presence of sex hormones, confirming in vivo findings and supporting the validity of this in vitro model. Results from the proteomic profiling will further help clarifying the underlying dimorphic mode of action of CPF.

P158
Thyroid hormone-disrupting potential of wastewater treatment plant effluents across Europe: insights from an in vitro bioassay battery
ABSTRACT #216

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Growing concern over contaminants in freshwater ecosystems necessitates advanced analytical techniques for their detection and quantification. Wastewater treatment plant (WWTP) effluents are a major source of endocrine-disrupting chemicals (EDCs), which can interfere with thyroid hormone regulation. In this study, effluents from 20 WWTPs of varying capacities and treatment technologies across 15 European countries were collected during a sampling campaign using on-site large-volume solid-phase extraction. The resulting extracts were assessed in an in vitro bioassay battery targeting key thyroid-related pathways, including the sodium/iodide symporter (NIS), thyroid peroxidase (TPO), transthyretin (TTR) binding, thyroid receptor (TR) agonism and antagonism, and aryl hydrocarbon receptor (AhR) activation. Our findings reveal that multiple WWTP samples exhibited inhibitory effects on thyroid hormone pathways, with pronounced inhibition of NIS, TPO and TTR binding activity and AhR activation. This study provides new insights into the impact of European WWTP effluents and addresses previously understudied endpoints. The presence of EDCs with thyroid hormone-disrupting potential in WWTP effluents highlights the need for integrated effect-based and chemical analytical approaches to improve the assessment and regulation of

environmental contamination. The project has received funding from the EXPRO GAČR No. GX20-04676X .

P159
Advancing mechanistic investigations of thyroid toxicity through new analytical parameters in a 3D RAT thyroid model
ABSTRACT #234

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Assessing disruptions in the thyroid hormonal system due to developing exogenous substances is crucial for ensuring product safety, especially in agrochemicals. New approach methodologies (NAMs) offer innovative opportunities to enhance our understanding of underlying mechanisms while reducing animal usage in research (the 3Rs). In this context, we used a 3D rat thyroid model based on primary thyrocytes cultured in collagen drops. Our multidisciplinary approach combined immunostaining, histology, automated confocal microscopy imaging, and pathological analysis to detect morphological and functional anomalies. We evaluated thyroid hormone (TH) levels in parallel to gain insights into affected biochemical pathways, providing a holistic view of organ status. We developed an automated confocal microscopy protocol for our 3D model, enabling enhanced imaging capabilities. The integration of automated phenotyping through high-content confocal imaging allows for the assessment of morphological responses. Additionally, multiplexing of thyroid biomarkers facilitates a comprehensive analysis of thyroid-related processes, enhancing our understanding of their biological significance. Morphological findings indicate that our rat thyroid model retains its native follicular architecture, including colloid with thyroglobulin and apical and basolateral cell poles. Immunohistochemistry (IHC) confirmed the expression and localization of markers involved in hormone synthesis, such as thyroperoxidase (TPO) and thyroglobulin, as well as transporters like MCT8 and highlighting the presence of C cells involved in calcium regulation. Functional analysis confirmed inducible thyroid hormone secretion in response to TSH and assessed TH secretion inhibition related to precursor depletion or excess, along with the use of NIS and TPO inhibitors. This 3D rat thyroid model, combined with our morphological and functional analysis protocol, is designed to identify hazards associated with endocrine disruptors and clarify their direct effects on the thyroid, aiding in the development of new products and having significant implications for toxicology and metabolism research.

P160
Endocrine-disrupting potential of pollutant mixtures in settled dust samples from households with little children from eight European countries
ABSTRACT #239

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Humans spend most of their time indoors, where limited ventilation and multiple pollutant sources lead to exposure to complex chemical mixtures, many with endocrine-disrupting (ED) properties. Young children (≤5 years) are particularly vulnerable due to ongoing development and higher exposure from floor contact and hand-to-mouth behavior. Indoor settled dust was collected from households with young children in eight European countries (Czech Republic, Estonia, Italy, Netherlands, Portugal, Slovenia, Sweden, and the UK) and processed using exhaustive organic extraction. The ED potential of pollutant mixtures was assessed using a battery of in vitro bioassays prioritized according to an Adverse Outcome Pathway (AOP)-based strategy. These included human cell-based reporter gene assays targeting key receptors such as aryl hydrocarbon receptor (AhR), estrogen, androgen, and thyroid hormone receptors. A subset of samples underwent additional testing for thyroid hormone (TH) system disruption, covering endpoints linked to adverse neurodevelopmental effects, namely thyroperoxidase inhibition, TH transport via transthyretin (TTR), and TH metabolization via deiodinase I. Most of the dust samples exhibited ED activity, with anti-estrogenic and AhR-related effects being most frequent. Notably, all subset samples significantly

interfered with TTR-mediated TH transport. The study's findings revealed diverse patterns of impact, intensity, and primary mechanisms of action of chemical mixtures from settled dust in European households, as well as interesting trends in the type and degree of effects across countries. To contextualize these findings, observed bioactivities will be compared with data from iceberg modeling obtained using available toxicity potencies based on available data from the target and non-target chemical analyses. This project received funding from the European Commission Horizon Europe, HADEA, research, and innovation program under grant agreement Project No 101057499 - INQUIRE.

P162
Testicular organoids: a potential model to study the impact of endocrine-disrupting chemicals on steroidogenesis
ABSTRACT #266

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A global decline in sperm counts has been observed over recent decades. Exposure to xenobiotics, including pollutants, and pesticides, with endocrine-disrupting activity, is considered a key contributing factor. Addressing this issue requires reliable human-relevant models to identify male fertility disruptors. In this context, we investigated human testicular organoids (TOs) as a promising in vitro model to mimic steroidogenesis and detect endocrine-disrupting chemicals. Primary testicular cells from eight transgender women were cultured in a stem cell medium in microwells for up to 61 days. Cellular composition was analyzed by immunofluorescence staining. To characterize steroidogenesis in TOs, transcriptome profiling with microarray and qRT-PCR were performed to assess the expression of key markers for the steroidogenic pathway. Steroid hormone levels were quantified via HPLC/MS or ECLIA. Organoids were stimulated with human chorion gonadotropin (hCG) (1 IU/L, 4500 IU/L) to test hormonal responsiveness. The model compounds Forskolin (100µM and 1mM) and prochloraz (10µM and 100µM) were administered on days 14, 16, and 18, and viability was assessed using the CellTiter-Glo® 3D assay. Protein-level analysis (n = 3) confirmed the presence of steroidogenic Leydig cells characterized by expression of STAR, CYP11A1, HSD3B2, and CYP17, along with positive staining for luteinizing hormone receptor and androgen receptor. The expression of steroidogenesis-related genes in TOs (n=3) was also confirmed at the transcript level. Testosterone peaked around day 14 (150.46 ± 105.80 ng/mL) and then stabilized, while estradiol remained constant (21.20 ± 2.92 ng/mL) (n=8). Organoids showed dose-dependent responses to hCG stimulation (n=3). Forskolin significantly increased testosterone, while prochloraz significantly reduced both testosterone and estradiol at both concentrations tested, without affecting viability (≥80%) (n=3). Testicular organoids recapitulate key features of human steroidogenesis and respond predictably to hormonal and chemical stimuli, supporting their use as a potential model for assessing EDCs and male reproductive toxicity.

P163
Hazard assessment of endocrine disruption potential using new alternative methods[SV1.1]: integration of in vitro thyroid peroxidase inhibition potency and metabolic fate of 3-amino-2,6-dimethylphenol explains its lack of thyroid toxicity in vivo
ABSTRACT #268

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NAMs for endocrine disruption (ED) assessment rely on three pillars: an endocrine mode of action, adverse effects in organisms and (sub)populations, and a biologically plausible link between the two. To date, this has required animal models for determination of adversity. We present a NAM-driven workflow for the hair dye 3-amino-2,6-dimethylphenol (Methylpurpur, MeP), integrating QSAR analysis, human toxicokinetic measures and modeling and thyroid peroxidase (TPO) inhibition profiling to characterize both parent compound and metabolites. This approach demonstrates how NAMs capture toxicokinetic and mechanistic information essential to infer endocrine hazard without recourse to additional animal studies. Toxicokinetic stability of MeP was assessed using human keratinocytes (dermal metabolism) and hepatocytes (systemic metabolism), enabling identification of all significant metabolites. QSAR modeling predicted potential (anti-)estrogenic, anti-androgenic, thyroid, or steroidogenic activities. TPO inhibition emerged as the principal concern. Relevant metabolites were synthesized and tested in vitro using the tyrosine iodination assay. Parent MeP showed inhibition potency comparable to propylthiouracil, but phase II mono-conjugation markedly reduced activity, and the di-conjugate was inactive, suggesting nucleophilic amino and phenol groups are required. In silico predictions aligned with in vitro data, suggesting metabolism plays a detoxifying role by attenuating activity. Our findings indicate that at systemically available levels of MeP and its metabolites, TPO inhibition is unlikely. This conclusion is corroborated by a 13-week OECD 408 rat study, which revealed no alterations in thyroid follicular morphology or T3, T4, and TSH hormone levels at doses up to 630 mg/kg bw/d. Collectively, these results indicate no relevant endocrine hazard from MeP and support the robustness of NAM-based ED hazard assessment. The workflow illustrates how NAMs can provide regulatory confidence in ED evaluation, reduce reliance on animal testing, and strengthen mechanistic understanding of chemical safety.

P164
Diverging endocrine disruption potential of resorcinol and paracetamol: thyroid peroxidase inhibition potency and metabolic fate differences under pharmaceutical and cosmetic use explain their contrasting endocrine disruption capabilities
ABSTRACT #271

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The evaluation of endocrine disruption (ED) hinges on three pillars: an endocrine mode of action, adverse effects, and a biologically plausible link. To date, this has required animal models for determination of adversity. Here, we demonstrate a NAM-driven approach which demonstrates the difference in thyroid disruption response from pharmaceutical and cosmetic uses of resorcinol (Res) and paracetamol/acetaminophen (APAP) or its dermal precursor p aminophenol (PAP). For this purpose, we leveraged the toxicokinetic stability of Res, PAP and APAP in keratinocytes (dermal metabolism) or hepatocytes (systemic metabolism) to determine free plasma concentration (Cfree). QSAR modeling was applied to predict the potential of these substances and their metabolites for (anti-)estrogenic, anti-androgenic, thyroid- or steroidogenic activities. TPO inhibition was identified as the main effect of concern. Parent substances and metabolites were tested for TPO inhibition activity using the in vitro tyrosine iodination assay. This use of QSAR, toxicokinetics, and modeling for pharmaceutical and cosmetic exposure scenarios clarifies when endocrine activity becomes biologically meaningful while adhering to 3R principles. Our findings highlight the importance of first-pass metabolism, with detoxification via N acetylation followed by O glucuronidation and sulfation. Res inhibited TPO only under specific high-exposure pharmaceutical conditions, not cosmetic use. Neither APAP nor PAP inhibited TPO in vivo, as parent and metabolite concentrations failed to reach biologically significant thresholds under either oral or dermal exposure. Altogether, these results emphasize the necessity of integrating

toxicokinetic data into hazard assessment using non-animal testing. The study demonstrates (a) the validity of NAMs for ED hazard evaluation and (b) the critical need to combine potency and metabolism data to ensure scientifically robust conclusions about the human health relevance of non-animal hazard findings.

P165
Monitoring PFAS and estrogenic bioactivity in source water using ERα- and PFAS-CALUX: A NAMs and ecosystem approach
ABSTRACT #273

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Background: Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants with potential endocrine-disrupting effects. Traditional chemical analyses quantify PFAS concentrations but often do not capture their combined biological activity. New Approach Methodologies (NAMs), such as in vitro CALUX bioassays, provide sensitive, animal-free tools to assess the functional impact of emerging contaminants. In this study, we applied both a PFAS-specific CALUX and an ERα-CALUX assay to source water, enabling detection of PFAS-related bioactivity and estrogenic activity prior to water treatment. The monitoring approach also considers local aquatic fauna, such as salamanders, to contextualize potential ecological relevance. Methods: Water samples were analyzed using the PFAS-CALUX assay and the ERα-CALUX bioassay, which employ human U2OS cells engineered with responsive reporters. Observations of local fauna were integrated to provide ecological context for potential endocrine-disrupting effects. Results: Preliminary analysis indicated that some water samples exhibited measurable PFAS-associated and estrogenic bioactivity. These observations suggest that combining PFAS- and ERα-CALUX assays can provide complementary information to standard chemical analyses while incorporating ecological considerations. Conclusion: Using PFAS- and ERα-CALUX assays exemplifies how NAMs can provide mechanism-based evaluation of emerging contaminants in source water. Integrating bioassays with ecological context enhances early detection of potential endocrine-disrupting chemicals, supporting both public health and environmental protection.

P166
Generation of a historical control database, and acceptance and interpretation criteria for an in vitro comparative T4-glucuronidation assay using primary human and RAT hepatocyte (TruVivo®) for determination of human relevance of liver enzyme-mediated thyroid effects
ABSTRACT #277

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Liver-mediated increased thyroid hormone (TH) metabolism is a frequent occurrence in rat studies and one which requires further assessment under the European 2018 ECHA-EFSA guidance on the identification of endocrine disruptors to determine the relevance to humans. Determination of the human relevance of such findings is posing significant challenges for applicants and regulators alike. New Approach Methodologies (NAMs) offer a promising path forward by enabling direct comparisons between human and rat responses. Among these, the comparative in vitro hepatic enzyme assay is a key NAM highlighted in the ECHA-EFSA

guidance. However, despite being listed in the finalised guidance, the development and implementation of this assay have proven technically complex and difficult to standardise. This study utilised TruVivo®, a tri-culture hepatic model comprised of hepatocytes and feeder cells (i.e., growth-inactivated fibroblast and epithelial cells), to compare T4-glucuronide (T4G) formation between human and rat systems. T4G serves as a proxy for TH metabolism and a critical endpoint in assessing human relevance of liver-mediated thyroid disruption. Here we describe the work conducted to characterise the variability of the model and endpoint, under basal and induced conditions, and between species. Furthermore, an Historical Control Database (HCD) was generated and an initial acceptability criteria developed. Additionally, this work aims to build confidence in the TruVivo® T4G assay as a reliable tool for evaluating human relevance for liver-mediated thyroid perturbation within the EU endocrine disruptor regulatory assessment framework.

P167
Functional and safety assessment of estradiol and phytoestrogens in human epidermal models
ABSTRACT #279

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Background: Genistein and daidzein are phytoestrogens with estrogenic activity. The SCCS Final Opinion (2022) concluded they are safe in cosmetics at concentrations up to 0.007% and 0.02%, respectively. Our study evaluated their epidermal effects across a concentration range including these levels and higher doses, in line with the EU call for data on endocrine disruptors in cosmetics. Methods: We used HaCaT cell line and in vitro reconstructed skin model (EpiDerm), viability (MTT), transcriptomics, permeability (using HPLC-MS) and activity (CALUX ERα bioassay) were assessed. Results: Treatments did not significantly impact viability. Transcriptomics revealed regenerative signatures with differences in immune and detoxification pathways. Crucially, although measurable amounts of estradiol, genistein, and daidzein permeated the epidermis, none of them activated ERα in CALUX. Conclusions: The absence of CALUX activation at permeating concentrations provides a pivotal safety indication, reinforcing SCCS conclusions and supporting in vitro models as robust tools for regulatory evaluation of endocrine-active cosmetic ingredients.

P168
Luteolin promotes steroidogenesis in I-10 mouse leydig cells exposed to atrazine
ABSTRACT #292

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Background_and_Objectives: Testosterone, the primary male sex hormone, is essential for male fertility and overall health. About 95% of total testosterone is synthesized by testicular Leydig cells via steroidogenesis. Disruption of this process by factors like pesticide exposure

can lead to testosterone deficiency. Atrazine (ATZ), a widely used herbicide, can impair steroidogenic function, while luteolin (Lut), a flavonoid, exhibits antioxidant properties. This study examines luteolin's effects on ATZ-induced dysfunction in I-10 Leydig cells by assessing viability, steroidogenesis, and gene expression. Material_and_Methods: Mouse Leydig I-10 cells were treated with DMSO (control), ATZ (3.3, 10, 33, and 100 µM), Lut (1, 3.3, 10, and 33 µM), and a co-treatment of ATZ + Lut (ATZ 33 µM + Lut 3.3 µM or ATZ 100 µm + Lut 3.3 µM) for 2, 4, and 6 days, with medium/chemical change every two days. Cell viability was measured at each time point, with testosterone (T) and progesterone (P4) levels quantified by ELISA, and the expression of key steroidogenic genes (StAR, Cyp11a1, Hsd3β6, and Hsd17β3) analyzed by RT-qPCR. Results: ATZ alone reduced cell viability in a concentration-dependent manner but temporarily increased T levels. Lut alone enhanced both P4 and T production at higher concentrations (10 and 30 µM). In the co-treatment, Lut protected I-10 cells from ATZ-induced cytotoxicity and transiently stimulated both P4 and T production. Specifically, the ATZ 100 µM + Lut 3.3 µM co-treatment showed the strongest effects, with P4 and T rising 2.6-fold and 1.9-fold, respectively, on Day 2, before returning to baseline by Day 6. These findings suggest co-exposure of Lut and ATZ may confer modest stimulation on steroidogenesis in I-10 cells. Discussion_and_Conclusion: In I-10 mouse Leydig cells, luteolin promoted survival and steroidogenesis in the presence of atrazine, suggesting a modulatory interaction that benefits I-10 cell vitality and enhances its steroid production.

P169
Multiple mechanisms of AhR/CAR/PXR activators in thyroid (T-) modality: an in vitro perspective on human relevance
ABSTRACT #300

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Thyroid hormones (THs) regulate metabolism, growth, and neurodevelopment. Xenobiotics may disrupt TH homeostasis through multiple molecular initiating events (MIEs), eg inhibition of the sodium/iodide symporter (NIS), thyroid peroxidase (TPO), and deiodinases (DIO1-3) as well as transthyretin (TTR). In parallel, activation of hepatic nuclear receptors such as the Aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) can induce phase I (CYP) and phase II (UGT) enzymes, enhancing hepatic clearance of conjugated THs, primarily via glucuronidation. Determining the human relevance of these effects remains a regulatory challenge. This study applies multiple new approach methodologies to evaluate both thyroid-specific and liver-mediated pathways. A panel of test substances, including AhR, CAR, and/or PXR activators, with or without documented effects on human serum TH levels, was assessed for inhibition of NIS, TPO, DIO1-3, and TTR, as well as for indirect liver-mediated thyroid effects. NIS activity was measured in HEK293 cells stably expressing human NIS. TPO activity was assessed in human thyroid microsomes. DIO1-3 were evaluated in HEK293 cells expressing each isoform, with T3 and rT3 quantified by LC-MS following T4 incubation. TTR-binding inhibition was evaluated via quenching of FITC-T4 fluorescence. Liver-mediated effects were investigated in cryopreserved rat and human hepatocytes (2D-sandwich, 7-days), measuring CYP1A, CYP2B, CYP3A, and UGT expression and activity, as well as T4 metabolism and clearance. Consistent with previous results, 5/9 test substances with reported TH exhibited multiple mechanisms of action. Liver enzyme induction alone did not explain the observed TH alterations. Further work will clarify hepatic contributions and TTR-binding inhibition, linking these findings to established thyroid MIEs. Overall, these findings illustrated that AhR/CAR/PXR activators can modulate TH homeostasis through diverse mechanisms, including direct effects on TH synthesis, transport, and metabolism. Combining these assays refines thyroid (T-) modality testing strategies and improves human relevance assessments.

P170
Interlaboratory validation study of a mineralocorticoid receptor transactivation assay (MRTA) to

identify endocrine disruptors in an in vitro setting
ABSTRACT #327

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Endocrine signaling pathways play a pivotal role in developing and maintaining the human body. However, testing and assessment of endocrine disruptors focuses predominantly on estrogen, androgen, thyroid, and steroidogenic (EATS) modalities. Other signaling pathways such as the glucocorticoid, retinoic acid, or mineralocorticoid receptor pathways are not included in current test guidelines. To fill this gap, validation and integration of new approach methods into test guidelines is urgently required. Here, we present the validation of the mineralocorticoid receptor transactivation assay (MRTA). Mineralocorticoids regulate processes such as electrolyte homeostasis and blood pressure. Moreover, disruption of mineralocorticoid signaling is implicated in the development and progression of kidney and cardiovascular diseases. The MRTA utilizes human U2OS cells that stably express a GAL4-hMR protein consisting of the DNA-binding domain of the yeast GAL4 transcriptional activator fused to the ligand-binding domain of the human MR. In addition, the cells contain a luciferase reporter gene controlled by GAL4-responsive elements. Therefore, luciferase expression is regulated by mineralocorticoids enabling the identification of activators and, in presence of the physiological mineralocorticoid Aldosterone, inhibitors of MR. To validate this method, a two-step validation with three participating laboratories is ongoing. During the transferability phase the method was transferred from the developer to two naïve laboratories. In addition, all laboratories tested six chemicals with known effects on MR. The results of all six chemicals showed good intra- and interlaboratory reproducibility and subsequently the ring trial phase was initiated. During the ring trial the three laboratories tested 30 blinded chemicals, which are either suspected MR agonists, antagonists, or neutral chemicals. The ring trial phase showed good reproducibility within and between the laboratories. After the final analysis of the data, a validation report will be prepared and submitted to the OECD for peer review, to ultimately adopt the method as a test guideline.

P171
Validation project of a fluorescent FITC-T4 transthyretin competitive binding assay
ABSTRACT #338

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Transthyretin (TTR) is the most important of the three plasma proteins distributing TH from the thyroid gland to the peripheral target tissues. Disruption of the binding interaction between thyroid hormones and TTR is considered a potential mechanism through which chemicals may interfere with the thyroid hormonal system. The method has already been assessed under the EU-NETVAL network for the pre-validation of thyroid assays, with a positive evaluation from the OECD thyroid disruption methods expert group which recommended further validation. The experts recognised TTR-binding as a relevant molecular initiating event for the identification and characterisation of thyroid hormone system disruptors. FITC (Fluorescein isothiocyanate) is a fluorescent probe which can be linked to thyroxine (T4). Upon binding of FITC-T4 to TTR, an increase in fluorescence is observed. This is the foundation for a competition binding assay where displacement of FITC-T4 from TTR leads to decreased fluorescence. The method is then used for testing of chemicals/compounds for their ability to compete for TTR binding. Competitors will reduce the fluorescence and for potent competitors the IC50 can be derived. Three naïve labs took part in the validation process (BASF in Germany, KaLy-Cell in France and VITO in Belgium). After training with the developers, the three labs started the transferability process with five reference compounds. They performed five independent runs which showed very good reproducibility and expected potency classification. Then a blinded ring-trail was performed with 18 coded substances, and the final results will be shown at the meeting.

P172
Enhancement project of the LC-MS/MS based steroidogenesis profiling assay (OECD TG456)
ABSTRACT #340

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The objective of this project is to reinforce the regulatory utility of the OECD TG456 for the H295R Steroidogenesis Assay by obtaining more comprehensive information concerning the disruption of steroidogenesis. The method is based on the publication by Ahmed et al. (2018) which utilized LC-MS/MS to measure the 19 distinct steroids of the steroid synthesis pathway. The project involves three laboratories (BASF, Germany, VU, The Netherlands and ANSES, France). Cells are incubated with control or test chemicals for 48 hours. During the transferability phase, three test items were tested with Forskolin and Prochloraz as controls. Nineteen hormones were quantified (including E2 and Testosterone the current readouts in TG456). Expected results were obtained, except for atrazine which gave negative results regarding its effect on Testosterone, while TG456 indicates it should be positive for Testosterone induction. This discrepancy seems to be a direct effect of the change in the Data Interpretation Procedure (DIP) in 2022 i.e. from 'statistical significance to solvent control, at 2 consecutive doses' to 'statistical significance AND 1.5 fold change compared to solvent control, at 2 consecutive doses'. As these results are in line with results obtained when the steroidogenesis assays was originally validated, a correction of the DIP in the OECD guideline was approved at the OECD WNT in April 2024. Furthermore, due to difficulties in assessing aldosterone, this hormone was removed from the panel of readouts. During the blind ring trial, the three labs have tested 15 compounds and assessed 18 steroids readouts. Results will be presented at the meeting.

P173
Chemical mixture driven androgen receptor disruption
ABSTRACT #352

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The ubiquitous presence of plastic nanoparticles (PNPs) in the environment raises concerns about their interaction with other pollutants. Due to the high specific surface area, PNPs can adsorb other environmental pollutants and facilitate their cellular uptake enhancing their toxic effects via so called "Trojan horse" action.1 Previous studies demonstrated already enhanced additive or synergistic effects of complex mixtures containing different PNPs and chemicals on cellular toxicity, proliferation, and inflammatory responses, whereas minimal or no effects were observed for individual components when tested alone.2 This study was focused on the androgen receptor (AR) response, which represents a critical target for assessing endocrine-disrupting effects related to development, metabolism, and reproductive function. For this purpose, we applied an OECD-aligned in vitro test guideline no. 458 to assess androgenic activity of differently sized polypropylene nanoparticles (PPNPs) and their mixtures with oxybenzone or methylparaben.3 AR-EcoScreen GR-KO M1 cells were used as a model system, as they stably express the human AR together with a luciferase reporter gene, enabling sensitive detection of receptor-mediated transcriptional activity. PPNPs (50 nm and 180 nm) were tested alone and in binary combinations with oxybenzone and methylparaben, which were also evaluated on their own. The results indicate that both sizes of PNPs, as well as oxybenzone and methylparaben individually, act as AR antagonists at the highest tested concentrations. Mixture exposures resulted in enhanced antagonistic activity compared to individual chemicals, suggesting a potentiation effect under combined exposure conditions.

P331
A NOVEL TPO-TRβ CALUX ASSAY FOR RAPID AND SENSITIVE DETECTION OF ENVIRONMENTAL

THYROID PEROXIDASE INHIBITING COMPOUNDS
ABSTRACT #480

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Thyroid peroxidase (TPO) is a key enzyme in thyroid hormone biosynthesis, responsible for catalysing the iodination and the coupling of tyrosine residues in thyroglobulin to produce thyroid hormones T3 and T4. Disruption of TPO activity can impair thyroid function and has been associated with endocrine disorders, including hypothyroidism and autoimmune thyroid disease. Bioassays are widely applied to screen for endocrine-disrupting chemicals, assess drug safety. Sensitive and reliable TPO bioassays are required to identify potentially harmful TPO disrupting compounds in complex environmental samples and to support regulatory assessment. Existing TPO inhibition assays use generic peroxidase substrates like luminol or Amplex UltraRed (AUR), producing light or color signals. These substrates however are not TPO specific and there are indications that substrate oxidation also occurs in the absence of active TPO proteins. Here we present the TPO-TRb bioassay, a novel TPO inhibition assay which measures the inhibition of T3 and T4 hormone formation from thyroglobulin. HEK-293 cell lines were transfected with the human TPO gene for stable and high TPO synthesis. Multiple HEK-293-TPO clones were compared to the HEK-293 wildtype and the clone with the highest and most consistent TPO production was selected. The TPO-TRb assay works by incubating TPO with its natural substrate thyroglobulin, with or without an inhibitor, after which the remaining thyroglobulin is completely digested. Generated T3 and T4 is subsequently measured using the TRb-CALUX luciferase assay and the level of TPO production is determined using the luminescent signal. The TPO-TRb assay shows consistent inhibition by its positive control MMI and has been tested and compared to the existing AUR TPO bioassay using a wide range of positive and negative compounds including the 31 compounds used in the official NETVAL thyroid validation study. This assay provides a physiologically relevant tool for assessing TPO inhibition, enabling better prediction of thyroid-disrupting potential.

Models, biomarkers and assays for systemic and immune toxicity

P174
In vitro genotoxicity assessment of chitin intended for use in the food industry
ABSTRACT #142

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Chitin, the second most abundant natural polysaccharide in nature, found in insects, fungi and crustaceans, represents a promising candidate for developing sustainable food contact materials for the food industry. However, safety evaluation is a crucial regulatory requirement before any consumer product can be authorized. Following the battery of assays recommended by the European Food Safety Authority for food contact materials, this study aimed to investigate the potential genotoxicity of shrimp shell chitin. Thus, a cytotoxicity assay was first performed on the L5178Y TK+/− cell line, both in the absence and presence of the S9 metabolic fraction. The tested concentration range was from 1.56 to 200 µg/mL. Subsequently, the genotoxicity potential of chitin was assessed using the in vitro micronucleus (MN) test in accordance with OECD guideline 487, to detect both clastogenic and aneugenic effects after 6 h (in the presence and absence of S9) and 24 h of exposure (in absence of S9). As no cytotoxicity was observed, the MN test was conducted using the same concentration range (up to 200 µg/mL). In the MN assay, both the cytokinesis block proliferation index (CBPI) and the frequency of binucleated cells with micronuclei (BNMN) were quantified. These parameters remained comparable to the negative control group at both short (6 h) and long (24 h) exposure times, indicating no observable effects at any tested concentration. A significant increase in positive controls was observed, which confirmed the validity of the assays carried out. Additional studies, such as the Mouse Lymphoma Assay, are being conducted to have a full picture of its genotoxic profile in vitro. Keywords: Chitin, micronucleus test, in vitro, genotoxicity.

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P175
Evaluating assay sensitivity in NGRA: A comparative study of cellular stress panels
ABSTRACT #143

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Historically, toxicological risk assessments relied on a consistent combination of standardised in vivo methods. The transition to next generation risk assessment (NGRA), however, is enabled by new approach methods (NAMs) that capture specific (e.g. receptor interactions) and non-specific (e.g. cellular stress) effects within a hypothesis-led framework. Non-specific NAMs are integral to the protectiveness of NGRA frameworks, though the combination and methods used in case studies varies. With NGRA frameworks allowing diverse assay combinations, understanding how assay choice impacts bioactivity characterisation is essential to ensure reproducibility and confidence in NGRA. To explore this, we compared two assays used in recent studies that characterise perturbation of cellular stress pathways, the “cell stress panel” [1] and “ToxProfiler” [2] using existing and newly derived data. The first tier compared assay characteristics (e.g. protocols, validation) which revealed similarities (e.g. cell lines, culture periods) and differences (e.g. biomarker/pathway coverage, dose-response assessment) that may affect the likelihood of identifying concordant bioactivity profiles. This was complemented by the second tier, through replicate testing of 40 chemicals, revealing qualitative and quantitative differences. These were interpreted alongside findings from the first tier, offering insights into assay-specific strengths, limitations, and implications for reproducibility in flexible NGRA frameworks.

P176
Integrated in vitro approach for immunotoxicity assessment of synthetic amorphous silica nanomaterials
ABSTRACT #169

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Synthetic amorphous silica nanomaterials (SAS-NMs) are widely used in food, cosmetics, and pharmaceuticals. Although considered “generally recognized as safe”, several studies have reported their ability to trigger pro-inflammatory and adjuvant-like responses. In line with the 3Rs principle, the establishment of New Approach Methodologies (NAMs) and Integrated Approaches to Testing and Assessment (IATA) is essential to evaluate such immunotoxicity without relying on animal models. This study aims to combine advanced physicochemical characterization with cellular and mechanistic endpoints to assess the immune-modulating potential of SAS-NMs differing in surface chemistry and specific surface area. Cytotoxicity and expression of co-stimulatory molecules were evaluated in human monocyte-derived DCs (moDCs) (CD80, CD83, CD86) and in the monocytic THP-1 cell line (CD54, CD86, CD40) using flow cytometry after 16h or 24h exposure. Allogeneic co-cultures of moDCs and $CD4^+$ T cells were used to assess T cell proliferation (CFSE assay) and activation (intracellular cytokines production) following moDCs priming with SAS-NMs. Our findings revealed that SAS-NMs with isolated silanol and high specific surface area induced significant phenotypic modifications of both moDCs and THP-1 cells. Reducing the silanol density led to a decrease of DC and THP-1 cells phenotype alteration. In agreement with the results obtained with DCs, $CD4^+$ T-cell

proliferation was slightly reduced when moDCs were pretreated with thermally treated SAS-NMs, compared to the native SAS-NMs (example for one donor : CFSE^low^ T cells = 35.4% for calcined vs 38.9% for native SAS-NMs at a 1:10 DC/T-cell ratio). These results demonstrate that dendritic cell-based NAMs can discriminate nanomaterials according to surface reactivity and immune activation potential. Our work contributes to the development of predictive, mechanism-based IATA frameworks for nanosafety assessment and supports the “safer-by-design” concept for industrial innovation.

P177
Toxicity and vector role of micro- and nanoplastics with pesticide residues in human cell line models
ABSTRACT #172

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Background and Objectives: Micro- and nanoplastics (MNPs) are increasingly recognised as emerging contaminants throughout the food chain, yet non-standardised detection methods limit accurate assessment of human exposure and health risk. Beyond their intrinsic toxicity, MNPs may act as vectors transporting environmental chemicals, including pesticides, into human tissues. However, the cellular effects of these exposures remain poorly characterised. This study investigated the toxicity of polystyrene (PS) and polyurethane (PU) MNPs, along with the pesticide residues imidacloprid and dimethomorph, in human liver (HepG2) and neuronal (SH-SY5Y) cell models. Each contaminant was examined independently to compare responses to commonly studied versus understudied plastics and pesticides, and to evaluate the potential of MNPs to enhance pesticide-associated cellular stress. Material and Methods: High-content screening (HCS) was employed to assess cell number, nuclear morphology, mitochondrial function, and other cytotoxicity endpoints. Initial experiments focused on 30 nm PS nanoparticles (5 and 10 µg/mL) applied to HepG2 cells for 24 hours. This has been expanded to PU particles and both pesticides in HepG2 and SH-SY5Y cells, as well as combined exposures to investigate vector effects. Results: Exposure to 30 nm PS nanoparticles resulted in a dose-dependent loss of HepG2 cell viability, with reductions of ~37% at 5 µg/mL and ~65% at 10 µg/mL. Nuclear area decreased by ~26–27%, indicating cellular stress. Mitochondrial mass declined by 18–20%, while membrane potential increased by 13–22%, suggestive of mitochondrial activation and oxidative stress. Nuclear intensity remained unchanged. Discussion and Conclusion: These findings demonstrate that PS nanoparticles induce significant cytotoxic and mitochondrial stress responses in human liver cells. Comparative studies with PU and pesticide residues, including co-exposure models, are clarifying the role of MNPs as vectors for pesticide transport and enhancing a mechanistic understanding of the impacts of emerging environmental contaminants at the cellular level.

P178
Investigation of the potential of Bisphenol A substitutes to induce allergic contact sensitization using OECD defined approaches
ABSTRACT #194

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Bisphenol A is a high production volume chemical used extensively in the manufacture of polycarbonate plastics, epoxy resins, and thermal printer paper. There is a high potential for occupational dermal exposure in the manufacturing of plastics, epoxies, and thermal paper, as well as post-production in the use of epoxy resins. In addition, bisphenol A containing plastics were commonly used in food packaging resulting in significant public exposure through leaching into food stuffs. The public is also at risk of dermal exposure due to environmental contamination. Mainly due to endocrine disruption effects, efforts have been made to replace bisphenol A with safer alternatives. Bisphenol A is known to be a

human skin sensitizer, particularly prevalent in occupational settings. However, there is a paucity of information available on the sensitizing potential of structural analogues which are increasingly being employed as substitutes. In the current studies, we utilized New Approach Methodologies (NAM) addressing key events 1-3 of the adverse outcome pathway for skin sensitization to address the potential of bisphenol A substitutes to induce dermal sensitization. Defined Approaches (DA) were used to further classify and categorize potency according to OECD TG 497. The NAMs and DAs confirmed that bisphenol A was a skin sensitizer in potency category UN GHS 1B. Bisphenol AF, bisphenol B, bisphenol AP, and bisphenol E were also classified as UN GHS 1B sensitizers, while 2,4-bisphenol S and bisphenol F were borderline sensitizers, and bisphenol S was classified as a non-sensitizer. Overall, these data, generated using NAMs and DAs, provide evidence that the structural analogues evaluated, except for bisphenol S, still pose risks for skin sensitization. This work was supported by the National Institute of Environmental Health Sciences, National Institutes of Health, U.S. Department of Health and Human Services contract HHSN273201400017C (Burleson Research Technologies, Morrisville, NC).

P179
Developing a 3D multi component human skin model for vitiligo biomarker identification
ABSTRACT #196

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Background and Objectives: Vitiligo is an autoimmune skin disorder affecting approximately 70 million people worldwide. Current research lacks robust, human-relevant in vitro models that can replicate the complex epidermal and immune interaction that leads to melanocyte destruction seen in vivo. In this study, we aimed to establish a 3D full thickness skin model incorporating immune cells to identify predictive vitiligo biomarkers following exposure to the benchmark depigmenting agents. Material and Methods: A multicomponent 3D skin model was constructed using immortalized N/TERT-2G keratinocytes and immortalized PIG1 melanocytes in a collagen hydrogel system. This model was adapted to incorporate THP-1 derived macrophages to mimic the dermal immune component. Models were exposed to several prominent depigmenting agents such as Hydroquinone, PPD, Rhododendrol and 4-TBP. Immunohistochemistry and qRT-PCR were performed to confirm melanocyte loss and elevate expression of inflammatory markers within the tissue constructs. Results: All the depigmenting agents induced expression of inflammatory cytokines including TSLP, IL8 in the 3D skin model in the presence of THP-1. We observed a similar phenomenon when the 3D skin models were treated with conditioned medium collected from PIG-1 cells treated with depigmenting agents in a ROS-dependent manner. This elevated expression of the inflammatory cytokines was not observed in the 3D skin model treated with other inhibitors of melanogenesis commonly used in cosmetic formulations such as ascorbic acid or kojic acid. Discussion and Conclusion: THP-1 integrated 3D skin provides a reliable human-relevant in vitro model to evaluate the molecular mechanism of chemically-induced vitiligo. An elevated inflammatory cytokine signature was evident across multiple depigmenting agents and validates these as potential biomarkers for vitiligo that can be used for compound screening in a high-throughput manner.

P180
Setting up new approach methods to study B cell immunotoxicity using peripheral blood mononuclear cells
ABSTRACT #214

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In the field of food safety, there is a growing concern about potential links between immune responses and exposure to foodborne compounds such as PFASs, bisphenol A, and micro- and nanoplastics. Due to the limited predictivity of animal models in human immunotoxicity, the complexity of the immune system, and the lack of emphasis on the

immune system in safety assessments, the immunotoxic effects of foodborne compounds in humans often become apparent only after exposure has reached critical levels. To study the impact of xenobiotics on the immune system, improved in vitro models predictive of human responses are needed. Peripheral blood mononuclear cells (PBMCs) provide a valuable cell population to study immunotoxicity, as they comprise a relevant mixture of human immune cells thereby enabling interaction between different immune cell types. Moreover, the derivation of PBMCs from human blood allow investigation of interindividual variability. The present study aims to develop new approach methods for robust and reproducible B cell stimulation within PBMC populations across multiple donors. Initially, the most effective strategy to stimulate B cells was investigated using five different types of stimulation, mimicking either T cell-dependent or T cell-independent activation. B cell stimulation was assessed by quantifying antibody secretion and analyzing B cell subsets along with their activation status via CD69 and CD83 marker expression using flow cytometry. Reproducibility was subsequently evaluated in two independent studies using PBMCs from similar donors. Finally, effects of various immunosuppressive compounds on B cell stimulation were investigated. Overall, T-cell dependent stimulation elicited the most robust and reproducible B cell differentiation and activation across donors, which was most strongly inhibited by the JAK1 inhibitor Upadacitinib. Altogether, the current study established a reliable and reproducible in vitro approach to study B cell immunotoxicity, which may serve as a valuable tool for evaluating immunotoxic effects of foodborne compounds.

P181
Recapitulating immune-driven hepatotoxicity using a liver microphysiological platform
ABSTRACT #224

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With the U.S. Food and Drug Administration (FDA) roadmap promoting New Approach Methodologies (NAMs) for regulatory testing of monoclonal antibodies (mAbs), there is a need for models with greater complexity and human relevance, with immune-mediated drug-induced liver injury (iDILI) a significant concern in drug development. Human-specific immune system interactions with new modalities can lead to unpredictable hepatotoxicity, failing to be captured in traditional animal and high-throughput cell models. The FDA-recognised PhysioMimix® DILI assay incorporated peripheral blood mononuclear cells (PBMCs) to address this. Primary human hepatocytes were cultured under dynamic perfusion in Liver-12 plates by PhysioMimix Core forming 3D liver microtissues. These were analysed for functionality (CYP, albumin, urea) and clinically relevant injury markers (ALT, AST). Following four days of culture, HLA-matched PBMCs were added to the circulating media for a further four days and functionality assessed (cytokine profiling). Ipilimumab (anti-CTLA-4) or infliximab (anti-TNF-alpha) was dosed for 48 hours and samples were taken periodically post-dosing. Liver microtissues were evaluated for hepatotoxic responses while immune activation and inflammation was assessed by cytokine release. Immune cell interaction and recovery from the Liver-12 was completed to determine if PBMCs can transverse unhindered (60-70% recovery). Following co-culturing, maintenance of liver microtissue functionality and stable immune phenotypes was demonstrated. Upon dosing with mAbs, immune-mediated hepatotoxicity was detected with LDH, ALT/AST elevation, and reduction in functional biomarkers. Cytokine increases were only seen in ipilimumab-treated conditions, aligning with clinical data. This study demonstrates the ability of the PhysioMimix DILI assay to predict immune-mediated hepatotoxicity. Together, this assay provides a human-specific solution to the assessment of hepatotoxicity of large molecules. The FDA's endorsement of NAMs for investigational new drug (IND) applications marks a paradigm shift toward human-relevant, non-animal testing strategies, positioning the PhysioMimix® Core System as a pivotal tool for the future of immunotoxicity assessment and safer drug development.

P182
Development and characterization of an immunocompetent and vascularized

skin model
ABSTRACT #226

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Skin exposure to chemicals, cosmetics, drugs, and medical devices can induce sensitization leading to allergic contact dermatitis. Sensitization involves a series of key events (KE): the covalent binding of an hapten to skin proteins (KE1), the activation of keratinocytes (KE2) and dendritic cells (DCs; KE3) and the activation and proliferation of T cells (KE4). Although several in vitro tests have been proposed to predict the sensitization potential of chemical compounds, the recapitulation of key events into a physiologically relevant and reproducible cell-based model remains challenging. Here, we described the development of a three-dimensional immunocompetent and vascularized skin model based on immortalized cell lines to perform sensitization tests for medical devices, covering Key Events 1 to 3. The full-thickness skin model displayed the major structural and functional elements of human skin: a stratified epidermis made with N-TERT/2G keratinocytes, a dermis consisting of a collagen/fibrin hydrogel harboring BJ-5ta fibroblasts and HMEC-1 endothelial cells, and immune cells capable of detecting danger signals. The latter were derived from the THP-1 monocytic cell line, differentiated into DCs by GM-CSF and IL-4. THP-1-derived DCs expressed characteristic DC markers such as DC-SIGN and their activation with LPS induced a higher expression of the maturation marker CD86. A relevant structure of the epidermal and dermal compartments has been observed by microscopy, allowing topical application of sensitizers and irritants and the screening of inflammatory cytokines such as IL-6 and IL-8 in the culture supernatant. Altogether, the here-described immunocompetent and vascularized skin model could be one of the first in vitro models enabling the assessment of skin sensitization for medical devices, supporting the reduction of animal use for biocompatibility testing. Final validation will require direct comparison of the system with existing in vivo and in vitro tests.

P183
Comparative analysis of cellular respones to selected carcinogens in 3D HEPG2 monoculture and HEPG2/kupffer cell co-culture models
ABSTRACT #235

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In vitro hepatic cell models are regarded as a pillar of genetic toxicology. Traditionally, experiments are performed using 2D monolayer cell cultures, which, however, fail to replicate the structural and functional complexity of liver tissue. To overcome these limitations, hepatic 3D cell models, which have improved metabolic competence and enhanced expression of phase I and II xenobiotic-metabolizing enzymes have been developed. However, the majority of currently used hepatic 3D cell models are based on a single cell type, despite the liver being a multicellular organ composed of different cell types. In this study, we investigated differences in cellular responses to carcinogens with distinct mechanism of action in 3D monocultures of HepG2 cells and a 3D hepatic co-culture model consisting of HepG2 and liver-specific macrophage Kupffer cells (KC). Both 3D hepatic models were exposed to the carcinogens griseofulvin (GF; 2-200 µM) and cadmium chloride (CdCl2; 0.25-25 µM) for 24h. Cell viability was determined with the LDH assay, liver functionality by measuring albumin secretion, inflammatory responses by ELISA (IL-6, IL-8, TNFα) and genotoxicity using the comet assay. Both chemicals induced cytotoxicity at the highest tested concentrations in both cell models, albumin secretion remained unchanged regardless of the model or

compound tested. Both chemicals stimulated IL-8 release, which was more pronounced in the HepG2/KC co-culture model and after exposure to CdCl2, which triggered a dose-dependent response. In contrast, IL-6 and TNF concentrations were below the detection limit. $CdCl_2$ exposure caused an increase in DNA damage, as observed by higher tail intensity in the comet assay in both models. To further elucidate the underlying molecular mechanisms and potential differences between the models, gene expression analysis of DNA damage response (GADD45A, CDKN1A), repair pathways (ERCC1, RAD51) and xenobiotic-related genes (phases I and II) is being applied. Funded by HEU project CutCancer (101079113) and ARIS [P1-0245]

P184
Biodegradable plastics: contaminants of emerging concern
ABSTRACT #259

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Background: Biodegradable plastics such as polyhydroxybutyrate (PHB) and polybutylene succinate (PBS) are promoted as sustainable alternatives to conventional polymers. However, their toxicity and long-term effects remain unclear. This study established a toxicological baseline by examining cytotoxic, oxidative, and morphological responses of human HepG2 liver cells exposed to polystyrene (PS) nanoparticles. The aim was to determine whether biodegradability ensures safety or alters cellular stress pathways. Methods: HepG2 cells were treated with 30 nm PS nanoparticles at 5 and 10 µg/mL for 24 hours. High-content screening (HCS) assessed viability, nuclear morphology, mitochondrial mass, membrane potential, and reactive oxygen species (ROS). Fluorescence imaging and automated analysis quantified subcellular changes. The workflow was applied to Caco-2 and SH-SY5Y cells. Future transcriptomic profiling will target SOD2, COX5A, and IL-8, representing oxidative, mitochondrial, and inflammatory responses. Results: PS nanoparticles caused a dose-dependent cytotoxic and mitochondrial response. Cell number declined by 42% at 5 µg/mL and 91% at 10 µg/mL. Nuclear area decreased by 9–11%, consistent with chromatin condensation and apoptosis. Mitochondrial mass and membrane potential rose to 120% and 124% of control, indicating compensatory hyperpolarisation under oxidative stress. ROS increased, confirming oxidative imbalance. Imaging showed cell shrinkage and condensed nuclei. Conclusion: PS nanoparticles disrupt cellular homeostasis through oxidative and mitochondrial stress in HepG2. These findings define PS as a reference for assessing biodegradables. Future work will expand this framework to PHB and PBS nanoparticles for direct comparison of synthetic and microbial-derived materials.
Keywords: Microplastics, Biodegradability, Cytotoxicity, Oxidative Stress, Human Cell Models

P185
Linking structural similarity to toxicogenomic profiles: transcriptomic read-across of flavonoids
ABSTRACT #317

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Toxicogenomics (TxG) is a fundamental part of systemic toxicity assessment within state-of-the-art next-generation risk assessment frameworks. Because of its broad biological coverage, it also offers a data-rich basis for supporting read-across. To substantiate read-across based on TxG data, we investigated to which extend structural similarity of test compounds translates into comparable biological effects on transcriptome level. To this end, we exposed MCF7 cells to five non-cytotoxic concentrations of three structurally closely related flavonoids - apigenin, quercetin, and naringenin. Targeted whole-transcriptome sequencing (TempO-Seq) was performed after 6 h and 24 h exposure. Differential gene expression analysis revealed concentration-dependent transcriptome perturbations by all three tested compounds. Transcriptome changes were more pronounced after 24 h than after 6h for all three compounds. Apigenin showed the strongest transcriptome response on gene level, followed by naringenin and quercetin. This difference in potency was also reflected by the respective transcriptomic point of departures (tPODs). Differentially expressed genes showed moderate to high overlap

between compounds. The highest transcriptomic profile similarity was found between naringenin and quercetin (cosine similarity > 0.74). Pathway level analyses indicated shared perturbations, including estrogen responsive signaling, alongside compound specific differences in other biological processes. Overall, structural similarity between the three tested compounds was mirrored in their qualitatively similar transcriptomic responses, linking structure-based and transcriptomic read-across. These findings demonstrate how TxG can quantify similarities and differences of biological activity between structurally related compounds, supporting its use in mechanistically informed read-across.

P186
Assessment of the sensitizing potential of chemicals and proteins, alone and in mixtures, using the bmdc model
ABSTRACT #318

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Background: Occupational allergies represent a major occupational health issue. In many workplaces, exposures rarely involve a single substance but often result from multiple exposures with chemical and/or biological sensitizers. Although this situation reflects real working conditions, multiple exposures remain poorly documented in the literature and the most common hypothesis used by the regulatory authorities to estimate mixture effects is additivity [1]. Moreover, the Nrf2-Keap1 signaling pathway implicated in oxidative stress, is widely recognized as a regulator of inflammation [2]. However, the effect of multiple exposures on this pathway has not yet been investigated. An in vitro model based on the activation of bone marrow-derived dendritic cells (BMDC) has been developed and validated in our laboratory to assess the sensitizing potential of substances [3]. The objective of this study was to explore the sensitizing potential of mixtures compared with individual substances using the BMDC model. Methods: BMDC were exposed to four sensitizers: benzalkonium chloride (BC), N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine (NNDD), bovine beta-lactoglobulin (BLG), and hen's egg ovalbumin (OVA) alone or in binary mixtures. The activation of DCs was then evaluated by analyzing the expression of phenotypic markers by flow cytometry. The expression of Nrf2-Keap1 signaling pathway genes (Nrf2, Nqo1, HO-1, Srxn1,…) was measured by RTqPCR. Results: When tested individually, all substances induced a concentration-dependent increase in the expression of surface markers. Time-course experiments (16-72 hours) showed that 48 hours was the only common exposure time associated with dendritic cell activation. Genes involved in the Nrf2-Keap1 pathway were differentially expressed, depending on the substance. Under co-exposure conditions, the expression of membrane markers was generally higher compared with the expression induced with the individual substances. Conclusion: The significant effects observed after exposure to mixtures highlight the importance of taking multiple exposures into account and including them in risk assessment.
Keywords: Dendritic cells; sensitizers; mixtures; BMDC

P187
When safe isn't safe together: co-exposure to individually non-toxic compounds alters liver HEPG2 spheroid functional integrity
ABSTRACT #326

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Background and Objectives Three-dimensional (3D) liver models provide physiological relevance for evaluating chemical toxicity (1). This study investigated the effects of co-exposure to individually non-toxic concentrations of pharmaceuticals and

biopolymers in HepG2 3D spheroids using multiparametric endpoints. Materials and Methods HepG2 spheroids (~800 cells/spheroid) were generated in ultra-low attachment round-bottom 96-well plates. Day 5 spheroids showed functional characteristics under steady-state conditions. They were treated for 48 h with rifampicin, tamoxifen, ε-poly-L-lysine (ε-PL), hyaluronan (Hya), and poly-L-arginine hydrochloride (PAR70). Viability assessment identified toxic concentrations; Hya showed no toxicity. For co-exposure experiments, the highest non-toxic concentration was selected. The following combinations were evaluated after 48 h: rifampicin (50 µM)–tamoxifen (5 µM), Hya (1000 µg/mL)–ε-PL (100 µg/mL), Hya (1000 µg/mL)–PAR (20 µg/mL), and ε-PL (100 µg/mL)–PAR (20 µg/mL). Growth dynamics were monitored by imaging, and endpoints included viability, CYP3A4 activity, and biomarkers (AST, LDH). Results Rifampicin–tamoxifen co-exposure increased spheroid size at 24 and 48 h without affecting metabolic activity, CYP3A4, or LDH; however, AST was elevated, suggesting hepatocellular hypertrophy or mild membrane perturbation without overt cytotoxicity. Hya–ε-PL and ε-PL–PAR induced spheroid compaction and reduced metabolism. Both mixtures increased CYP3A4 activity, indicating metabolic adaptation or stress-induced upregulation. Neither combination altered AST, and LDH remained unchanged (ε-PL–PAR) or decreased (Hya–ε-PL), suggesting structural remodelling rather than membrane damage. Hya–PAR showed no differences from controls. Discussion and Conclusion Co-exposure to non-toxic concentrations of antibiotics or biopolymers induced subtle morphological and functional changes in 3D liver spheroids. Rifampicin–tamoxifen suggested sub-cytotoxic hepatocellular stress, while polymer-containing mixtures triggered adaptive metabolic responses, including spheroid compaction and CYP3A4 upregulation. These results indicate that individually non-toxic compounds may produce toxic effects when combined, as previously suggested (2), highlighting the importance of evaluating mixture toxicity rather than relying solely on single-compound assessments.

P188

Comparative roles of CYP2E1 in isoniazid- and iproniazid-induced hepatotoxicity
ABSTRACT #331

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Background and Objectives: Isoniazid (INH) is clinically used as an antituberculosis drug. Iproniazid (IPR), which has a similar structure to INH, has been used as an antidepressant based on its MAO inhibitory activity, but has since been withdrawn due to hepatotoxicity. Hepatotoxicity is a common adverse effect of both drugs but is considered more severe with IPR. In this study, we attempted to explain the similarities and differences between the two drugs through in vitro studies based on the recently proposed CYP2E1-mediated mechanism of INH hepatotoxicity. Material and Methods: CYP2E1 activity was measured using human, rat, and mouse liver microsomes (Ms). They were preincubated in the presence of NADPH with INH, IPR or their hydrazine metabolite, acetylhydrazine or isopropylhydrazine, followed by measurement of the residual activities. The covalent binding of reactive metabolites to CYP2E1 was evaluated based on the time-dependent CYP2E1 inhibition. Results: INH caused concentration-dependent inhibition of CYP2E1, which was enhanced by preincubation of Ms. This time-dependent inhibition suggested that CYP2E1 is a target in the development of hepatotoxicity. No major species differences in this inhibitory effect were observed in humans, rats, and mice. IPR showed similar inhibitory effects at lower concentrations than INH, corresponding to the severity of hepatotoxicity. Classically, it has been thought that hydrazine metabolites are directly involved in the hepatotoxicity of both drugs, but they showed inhibitory effects only comparable to those of the corresponding parent drugs. This suggests that reactive metabolites are generated directly from INH and IPR by CYP2E1 rather than via hydrazine metabolites. Discussion and Conclusion: INH and IPR are converted into reactive metabolites by CYP2E1 and haptenized CYP2E1 is thought to be involved in immune-mediated hepatotoxicity, with the effect being stronger in IPR. These results may be considered an example of toxicophores in drug-induced hepatotoxicity.

P189
Mechanistic investigation of the immunotoxicity of synthetic amorphous silica nanomaterials using new approach methodologies
ABSTRACT #341

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Allergies represent a major public health challenge, and the World Health Organization estimates that by 2050 they could affect more than half of the global population. Among environmental risk factors contributing to this increase, exposure to nanomaterials, particularly synthetic amorphous silica nanomaterials (SAS-NMs), remains insufficiently investigated despite their widespread use in everyday products, including food and pharmaceutical applications. Recent studies suggest that SAS-NMs have immunomodulatory properties, notably through the activation of dendritic cells, which may exacerbate allergic responses upon co-exposure with allergens (1, 2). However, early cellular and molecular mechanisms underlying this immune activation remain poorly understood. The aim of this work is to characterize the immunotoxic effects of SAS-NMs by identifying early key events and molecular initiating mechanisms (MIEs). Human dendritic cells (DC) and the THP-1 cell line (used as a surrogate model) are employed to investigate membrane perturbations and signaling pathway activation following SAS-NM exposure. These cellular approaches are complemented by studies on biomimetic membranes, including giant unilamellar vesicles (GUVs) and giant plasma membrane vesicles (GPMVs). The implementation of these simplified models allowed us to investigate the contribution of direct nanomaterial-membrane interactions while bypassing the complexity of the cell. Preliminary results show that exposure to SAS-NMs induces rapid plasma membrane reorganization, including lipid raft formation, together with early activation of kinase-dependent signaling pathways involving Src and Syk, supporting a membrane-driven mechanism of activation and confirming our hypothesis for MIE of SAS-NMs. Moreover, scanning electron microscopy of THP-1 revealed morphological alterations induced by SAS-NMs with cells covered by nanomaterials aggregates. GPMVs and compositionally relevant GUVs, along with fluorescent probes use, have been optimized, and the effect of SAS-NMs is under study. In addition, this work contributes to the development of human-relevant in vitro testing strategies for nanomaterial immunotoxicity in line with the 3Rs principles.

P190
Development of a large-pore cell-based in vitro intestinal model for size-dependent microplastic transport and safety assessment
ABSTRACT #353

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Background and Objectives Microplastics (MPs) and nanoplastics (NPs) are pervasive environmental contaminants, with human exposure occurring predominantly via ingestion. While nanoscale particle uptake has been extensively investigated, the intestinal translocation of larger microplastics remains poorly understood. This limitation is partly due to the constraints of conventional in vitro systems, where commercially available inserts typically possess pore sizes ≤ 8 μm, restricting the assessment of larger particle permeability. This study aims to evaluate size-dependent microplastic translocation using a non-conventional intestinal model designed to overcome these limitations. Materials and Methods A physiologically relevant co-culture model was established using Caco-2 intestinal epithelial cells and intestinal myofibroblasts cultured on a porous nylon mesh with 30 μm openings. This large-pore, cell-based membrane enables the investigation of particle transport beyond the constraints of standard

inserts. Fluorescent microplastic particles of defined sizes (1 µm, 12 µm, and 22 µm) were applied to the apical compartment, and their translocation across the epithelial barrier was quantified under controlled conditions. Results A clear size-dependent permeability profile was observed. Particles of 1 µm consistently translocated across the epithelial layer, whereas larger particles (12 µm and 22 µm) exhibited no detectable translocation. Compared to conventional insert systems, which allow limited passage of small particles but are structurally restrictive, the present model enables a more accurate assessment of size-dependent transport behavior. Discussion and Conclusion These findings demonstrate that microplastic translocation across the intestinal epithelium is highly size-dependent, with an apparent upper size threshold between 1 µm and 12 µm under the present conditions. Importantly, this study highlights the critical role of membrane architecture in permeability assessment and introduces a novel in vitro platform capable of evaluating larger particle transport. The model provides experimentally grounded constraints for microplastic uptake and offers valuable input parameters for future exposure modeling and risk assessment.

P191
Ex vivo nonclinical system using fresh circulating human whole blood (id.flow®) for evaluating immunotoxicity
ABSTRACT #371

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Animal models have limitations in translatability, and it is desirable to reduce the use of preclinical animals in accordance with the 3Rs. Therefore, it is important to have a physiologically relevant test system for predicting immunotoxicity. ID.Flow® is an ex vivo system that mimics the human blood circulation, in which fresh human whole blood, in the presence of minimal anticoagulant, is circulated at 37 °C together with test substances or clinically relevant controls. Samples can be analysed at several different time points, up to 24 hours. Multiple readouts, such as cytokine release and cell depletion, can be assessed from each sample. In contrast to static whole blood plate-based models that use high levels of anticoagulants and contain inactive cascade proteins, ID.Flow® measures complement activation, platelet aggregation, activation, and coagulation, similar to what would be observed in the body. Easily activated cells, such as neutrophils and basophils, are present (unlike in isolated PBMC models) but not disturbed in ID.Flow®, thereby keeping the background release of inflammatory cytokines low. ID.Flow® can be used for predicting infusion-mediated effects (e.g., cytokine release syndrome, allergic reactions, thrombocytopenia/neutropenia, haemolysis, clotting) of many drug modalities, including antibodies and antibody-like molecules, oligonucleotides, drug delivery vectors, and small molecules predicted to enter the bloodstream. The system has been in use for over 10 years, during which multiple clinical substances have been evaluated using both human [1] and patient blood [2], showing good correlations between ID.Flow® and clinical observations. Shown here are data from FDA-approved modalities, including oligonucleotides (imetelstat and inotersen) and antibody-based therapies (cetuximab, alemtuzumab, obinutuzumab, and glofitamab), where ID.Flow recapitulated their clinical responses, supported by comprehensive data on complement [3], cytokine, and cellular activation.

P192
Predictive and mechanistic immunotoxicology using single-cell metabolomics
ABSTRACT #402

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Immunotoxicology remains a major challenge in preclinical drug safety, with current in vitro assays offering limited mechanistic resolution. Single-cell metabolomics (SCM) is an emerging technology that captures the full metabolic state of individual immune cells, providing a rich, unbiased phenotypic readout. We recently developed high-throughput SCM methods

specifically optimized for T cells, enabling large-scale metabolic profiling at single-cell resolution that can be used for mechanism-resolved, AI-driven immunotoxicity prediction. We investigated whether SCM profiles of Jurkat T cells, combined with machine learning, can accurately predict immunotoxic mechanisms of action across five toxicity classes, and whether SCM-derived markers offer superior sensitivity to conventional viability readouts. Jurkat T cells were exposed to compounds spanning five immunotoxicity classes: genotoxic/DNA synthesis inhibition, oxidative/lipid peroxidation stress, mitochondrial dysfunction/bioenergetic stress, metabolic/nutrient deprivation, and non-toxic controls. SCM data were acquired using HT SpaceM, which computationally integrates microscopy and MALDI mass spectrometry imaging data. A machine learning method was developed incorporating dual-modality quality control, inter-slide batch correction, feature selection, and cell filtering. MoA prediction was evaluated by leave-one-treatment-out cross-validation. Across 44 evaluated treatments, the classifier achieved 57% top-1 accuracy, rising to 68% when accounting for compounds eliciting no cellular response, and 88% after mechanistically validated reclassification. Crucially, single-cell resolution revealed within-sample mechanistic heterogeneity: for gemcitabine, distinct cell subpopulations were predicted as genotoxic and mitochondrially dysfunctional — both supported by published evidence — a dual effect invisible to bulk assays. We will present single-cell metabolic analyses revealing mechanistic signatures per toxicity class and drug. A dose-response study shows SCM-derived markers detect toxicity below viability-affecting concentrations, with predicted toxicity classes shifting with dose. Overall, single-cell metabolomics combined with machine learning enables accurate prediction of immunotoxic mechanisms in T cells, outperforming viability-based approaches in sensitivity and mechanistic depth.

P193
Integrating complementary in vitro endpoints reveals mitochondrial and oxidative effects of PAβN beyond standard viability assays
ABSTRACT #404

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Background and Objectives: Phenylalanine-arginine β-naphthylamide (PAβN) is a widely used efflux pump inhibitor in microbiological research and antimicrobial potentiation studies. Despite its frequent use, it is often described as highly toxic, although quantitative data supporting this claim remain limited. This study aimed to characterize the concentration-dependent cytotoxic and mitochondrial effects of PAβN across multiple human cell lines. Material and Methods: Human immortalized bronchial epithelial (BEAS-2B), pulmonary (A549), renal (HEK293), and hepatic (HepG2) cell lines were exposed to increasing concentrations of PAβN. Metabolic activity, membrane integrity, and cell number were assessed after 24 h exposure. Early mitochondrial membrane potential changes (JC-1) and reactive oxygen species (ROS) production were evaluated after 1 h exposure. Results: Metabolic viability was not significantly altered across all tested cell lines. Membrane damage occurred predominantly at high concentrations approaching solubility limits, consistent with non-specific membrane perturbation. JC-1 analysis revealed a biphasic mitochondrial response in BEAS-2B, A549, and HEK293 cells, characterized by hyperpolarization at intermediate concentrations (250–600 µg/mL), followed by depolarization at the highest dose. In contrast, HepG2 cells did not exhibit hyperpolarization but showed depolarization only at 1000 µg/mL. Notably, ROS production was selectively increased in HepG2 cells at lower concentrations. Discussion and Conclusion: PAβN induces concentration-dependent and cell-type specific mitochondrial alterations that are not detected by metabolic viability assays. Early mitochondrial changes and hepatocyte-specific oxidative stress may precede overt cytotoxicity, while membrane damage appears mainly at high concentrations. These findings highlight the importance of integrating complementary and mechanistically informative endpoints for accurate in vitro toxicological assessment.

P194

Testing immunomodulatory effects (IMOC) of micro-and nanoplastic particles (MNP) and isothiazolinones in human monocytic cells
ABSTRACT #413

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Background: Chemically very different plastics, e.g. polystyrene (PS), polypropylene (PP), polyamides (PA), polyethylene (PE) and polyvinyl chloride (PVC) are used in consumer and medical products. Due to their composition, they have advantageous properties including moldability, relative lightness, chemical resistance and biocompatibility. Unfortunately, numerous decompose slowly and can remain in the environment for many years. Weathering factors (UV-radiation, temperature, mechanical stress) then generate micro-/nanoplastic-particles (MNPs). MNPs can be ingested through food and inhalation. In addition, MNPs can cross tissue barriers and affect cells. Hence, potential effects of MNPs in the immune system and in immune cells (e.g. monocytes, dendritic cells, T cells) are of great concern. Chemicals like isothiazolinones (e.g. 2-methyl-4-isothiazolin-3-one (MI), a sensitizer) are used as preservatives in cosmetics or as biocides e.g. in paints and some plastics. The method applied here is based on human monocytes from peripheral blood mononuclear cells (PBMC) and enables investigations of toxic immune effects. The cells and their cellular descendants play a pivotal role in antigen-processing, thereby linking adaptive and innate immune responses. By using multiparametric flow cytometric analyses (BD-FACS-Aria-III) the following parameter were examined: (i) monocytic subpopulation phenotyping (based on CD14, CD16), (ii) induction of cell surface markers (CD86, CD40, HLA-DR, PD-L1) and (iii) lysosomal protease cathepsin B (CatB) activity, potentially involved in T cell epitope editing. Results: (i) Subpopulation phenotyping: A clear shift from predominantly intermediate monocytes to classical monocytes can be observed for MI (concentration dependent; 5-500µM) as well as for PS100nm (not concentration dependent; 10-250µg/mL), compared to control. (ii) PS100nm significantly increased expression of all activation markers (CD86, CD40, HLA-DR, PD-L1); whereas MI induced only one marker. CatB activity was only affected by MI, at the highest concentration tests. Conclusion: Thus, IMOC testing may represent an additional tool in NAM immune toxicity analyses. (PolyRisk EU Grant Agreement No 964766; https://polyrisk.science/)

P195
A combined comet assay and viability strategy for phototoxicity and photogenotoxicity screening in HaCaT cells
ABSTRACT #436

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Ensuring the safety of UV-absorbing ingredients in drugs and skincare is essential for preventing skin irritation and cancer. However, current regulatory recommendations mainly rely on the 3T3 Neutral Red Uptake (NRU) assay, which detects photocytotoxicity but does not address photogenotoxicity. As a result, DNA damage occurring at sub-lethal concentrations may remain undetected, creating a regulatory gap. In line with the "3Rs" principles, there is a need for integrated in vitro approaches capable of simultaneously evaluating both endpoints. This study applies a streamlined, dual-endpoint screening model in HaCaT human keratinocytes to evaluate a diverse library of chemicals. Tested compounds included Benzophenone, Chlorpromazine, 8-Methoxypsoralen (8-MOP), Retinoic Acid, 6-Methylcoumarin, and p-Phenylenediamine (PPD), with Sodium Dodecyl Sulfate (SDS) and Lactic Acid as negative controls. HaCat cell cultures were

exposed to the compounds for 1 hour and then irradiated with 4 J/cm² of UV-A. To detect transient DNA damage before repair processes could occur, the alkaline Comet Assay was conducted immediately after irradiation using a medium-to-high throughput format. In parallel, photocytotoxicity was assessed 24 hours later in compound-free medium using the MTT assay, allowing calculation of Photo-Irritation Factors (PIF) and Mean Photo Effects (MPE). This combined analysis enabled clear differentiation between direct cytotoxicity and DNA damage occurring at non-cytotoxic concentrations. SDS, lactic acid and PPD did not show phototoxic responses, whereas the remaining compounds displayed clear phototoxic effects (PIF > 5 or MPE > 0.15). The method successfully detected substances that induce significant UV-dependent DNA damage, such as chlorpromazine. Interestingly, 8-MOP did not produce detectable DNA migration, likely due to its DNA crosslinking mechanism. This integrated strategy represents a rapid and ethically responsible tool for early-stage screening, facilitating the identification of both acute phototoxicity and otherwise undetected photogenotoxic risks. Grant PID2020-113186RB-I00 funded by MICIU/AEI/10.13039/501100011033 (Ministerio de Ciencia, innovación e universidades)

P332
Human iPSC-derived glial cells for functional inflammatory assays and toxicity testing
ABSTRACT #444

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Microglia and astrocytes are always activated in brain toxicities and neurodegenerative pathologies. However, data on compounds affecting glial inflammatory responses and cell-based platforms to test for this are scarce. Here, we developed an NFκB translocation assay to capture glial responses to inflammatory stimulation and their modulation through drugs or toxicants. We utilized human iPSC-derived microglia and astrocytes exhibiting glial-specific characteristics (Wolfbeisz et al., 2025; Spreng et al., 2022). NFκB translocation was selected as assay readout since many inflammatory pathways converge on this key event. Various TLR ligands and cytokines were applied to induce nuclear NFκB translocation, and optimal concentrations and time points were experimentally defined. Positive controls were established, and reference compounds, including IKK and proteasome inhibitors, were evaluated. Additional viability assays ensured non-toxic working concentrations. Our findings highlight the utility of the NFκB translocation assay in human glial cultures as a platform for screening compounds that modulate glial inflammatory signaling.

P333
COMMON METABOLIC DISRUPTIONS IN PSYCHOSTIMULANT-EXPOSED HEPARG CELLS: IMPLICATIONS FOR MITOCHONDRIAL FUNCTION AND TOXICOLOGICAL SCREENING
ABSTRACT #472

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New psychoactive substances (NPS), including synthetic cathinones and amphetamine-type stimulants, represent a growing challenge for toxicological detection and risk assessment. Their metabolic effects, particularly at the cellular level, remain incompletely characterized. This study aimed to identify common metabolic alterations induced by structurally diverse psychostimulants using the HepaRG cell model. Cell viability was first assessed to determine non-cytotoxic exposure concentrations. HepaRG cells were then

incubated with four psychostimulants (i.e., 3-MMC, alpha-PHP, MDMA, and methamphetamine). Time-course experiments were conducted with samples collected at multiple time points to monitor metabolic changes. Untargeted metabolomic analyses were performed using complementary HILIC and reversed-phase liquid chromatography coupled with high-resolution tandem mass spectrometry (ESI+ and ESI- HRMS/MS). Both intracellular extracts and culture supernatants were analysed to provide a comprehensive overview of metabolic alterations. Despite structural differences, all compounds induced shared metabolic changes. Notably, a consistent decrease in acylcarnitines, particularly long-chain species, was observed in both cells and supernatants. In addition, reduced levels of dicarboxylic acids were detected. These findings are consistent with a potential disruption of mitochondrial fatty acid metabolism. Such convergent metabolic signatures may represent valuable biomarkers for broad-spectrum toxicological screening of psychostimulants, including NPS. Overall, this study highlights the relevance of HepaRG cells for investigating drug-induced metabolic perturbations. Future work will involve the use of CYP2D6-overexpressing models to further refine the characterization of psychostimulant metabolism.

P334
HUMAN PRECISION-CUT LIVER SLICES AS AN IMMUNOCOMPETENT PLATFORM FOR DRUG INDUCED LIVER INJURY ASSESSMENT
ABSTRACT #483

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Background: Idiosyncratic drug-induced liver injury (iDILI) is characterized by delayed onset and immune-mediated mechanisms are thought to explain its idiosyncratic nature, whereas non-iDILI compounds typically induce dose-dependent acute hepatotoxicity. Current animal-free safety testing approaches mainly rely on single-cell models, which, although scalable and reproducible, lack multicellular interactions including crucial hepatic-immune interactions and tissue microenvironment. Human precision-cut liver slices (hPCLS) preserve hepatic cellular composition, tissue architecture, and donor-specific genetic variability, enabling the study of inter-individual differences. Here, we aim to characterize hPCLS as an in vitro platform for drug toxicity assessment and to investigate the contribution of immune cells to the susceptibility and onset of iDILI and intrinsic DILI. Methods: Tissue viability was assessed under different medium compositions and oxygen concentrations over 1–7 days of culture. Next, hPCLS were exposed for 24 hours to non-iDILI compounds (acetaminophen, valproic acid, 5-fluorouracil, chlorpromazine, and perhexiline) and IC_{50} viability values were extracted from concentration–response curves (n = 3). For iDILI-associated compounds (nevirapine, amodiaquine, troglitazone, and tolcapone), hPCLS were treated with three increasing concentrations for 24, 48, and 96 hours. Toxicity and inflammasome activation were evaluated by measuring ATP levels and IL-1β secretion, respectively (n = 3). Results: hPCLS cultured in WEGG medium supplemented with insulin–transferrin–selenium under 20% O_2 exhibited optimal viability for up to 7 days. Preliminary results demonstrated IC_{50} and viability profiles of intrinsic DILI drugs comparable to those reported for 2D and 3D hepatocyte cultures. IL-1β was detected in culture supernatants and showed concentration- and time-dependent responses to amodiaquine and troglitazone exposure. Discussion and Next Steps: Overall, our preliminary findings support the use of hPCLS as a physiologically relevant platform for iDILI assessment. Future studies will include a larger sample size and focus on immunophenotyping and transcriptomic profiling of relevant cell populations to identify immune-related mechanisms underlying hepatotoxicity.

Non-animal methods for safety testing of biopharmaceuticals/ biotherapies/vaccines

P196
Development of human iPSC-derived hepatocytes for drug discovery, translational research and toxicity testing
ABSTRACT #198

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Hepatocytes are responsible for key liver functions, including detoxification and xenobiotic metabolism, making them essential for in vitro toxicology. Despite their critical role, their use is hampered by challenges with traditional models, such as the scarcity and variability of primary human hepatocytes (PHHs), the poor physiological relevance and metabolic function of immortalised cell lines, and the poor scalability and high lot-to-lot variability of human induced pluripotent stem cells (hiPSCs) directed differentiation methods. These limitations hinder the ability to predict drug-induced liver injury (DILI), contributing to the 90% attrition rate of drug candidates in clinical trials and underscoring the need for better liver models. We used opti-ox™, a deterministic cell programming technology, to generate consistent, scalable, hiPSCs-derived hepatocytes, ioHepatocytes, and demonstrated their suitability for toxicology, research and disease modelling. ioHepatocytes display classic cobblestone morphology with distinctive nuclei and well-defined borders. Cells express key pan-hepatocyte markers including ALB, HNF4A, ASGR1 and SERPINA1, and present a transcriptomic signature similar to PHHs. Additionally, ioHepatocytes derived from hiPSCs with opti-ox™ demonstrate high batch-to-batch consistency. Functionally, ioHepatocytes perform critical hepatocyte functions including albumin secretion, glycogen storage, and accumulation of lipids. Most importantly, ioHepatocytes show expression of genes involved in phase I, II and III of drug metabolism and have functional cytochrome P450 enzymes (CYP3A, CYP2B6 and CYP1A2). When challenged with compounds known to cause toxicity, ioHepatocytes show a clear dose-dependent decrease in cell viability that strongly correlates with the known DILI severity of the compounds. Importantly, the toxic response very closely mimicked that of PHHs, validating the predictive capability of ioHepatocytes. ioHepatocytes offer a highly consistent, functional and scalable human hepatocyte model, overcoming the limitations of existing technologies. Their demonstrated ability to respond to toxic insults similarly to PHHs suggests their use as a reliable, predictive tool for improving DILI risk assessment in drug development.

P197
Development and validation of a human reporter cell-based assay for mitochondrial toxicity assessment ABSTRACT #222

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Mitochondrial dysfunction is increasingly recognized as a critical toxicological mode-of-action for both drugs and chemicals and is a common cause of drug attrition and post-market withdrawal. Reliable assessment of chemical-induced mitochondrial toxicity is therefore crucial for accurate chemical safety evaluations and risk predictions. Current screening approaches for mitochondrial toxicity often exhibit limited specificity and sensitivity, highlighting the need for more predictive testing strategies. A human cell–based reporter assay was developed to detect chemical-induced mitochondrial toxicity via live-cell confocal imaging. The assay employs the HepG2 CYC1-GFP reporter line, in which the CYC1 protein—a subunit of complex III of the mitochondrial electron transport chain (ETC)—is tagged with GFP, enabling direct visualization of the mitochondrial network in living cells. Mitochondrial morphology was quantitatively assessed using morphology parameters capturing fluorescence intensity distributions (using the “Symmetry-Threshold Compactness-Axial-Radial (STAR)” morphology methodology) and local texture patterns (using the “Spot-Edge-Ridge (SER) features” methodology) to distinguish structural phenotypes such as tubular versus punctate mitochondrial networks. The assay was validated using a panel of 20 known mitochondrial and non-mitochondrial toxicants (e.g., oligomycin, troglitazone, and cadmium chloride). Oligomycin, a complex V ETC inhibitor, induced mitochondrial fragmentation at a non-cytotoxic concentration (0.4 µM),

evident from the appearance of small punctate networks. In contrast, the mitochondrial structure was preserved upon exposure to the non-mitochondrial toxicant cadmium chloride. Overall, morphological assessment of mitochondria enables early and sensitive detection of chemical-induced perturbations in mitochondrial integrity and function, providing a robust morphology-based platform for improving the prediction of mitochondrial liabilities.

P198
Phenotypic screening platforms for neurotoxicity and neurorepair in CNS drug safety assessment
ABSTRACT #223

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The high attrition rate in CNS drug development underscores the need for predictive, mechanistically relevant in vitro models that support both efficacy and safety profiling. At Charles River, we have developed complementary phenotypic screening platforms using human iPSC-derived neurons to evaluate neurotoxicity and regenerative potential under disease-relevant conditions, aiming for alignment with recommendation frameworks like OECD for in vitro neurotoxicity testing. In one approach, we adapted the Cell Painting assay to profile morphological changes in ioGlutamatergic neurons either carrying pathogenic mutations or being exposed to neurodegenerative disease relevant triggers like βAmyloid. Cell Painting assays have been widely adopted for morphological profiling to uncover mechanisms of action, disease phenotypes, and toxicity prediction. A modified staining protocol incorporating β-III tubulin enhanced visualization of neuronal architecture. Principal Component Analysis revealed distinct phenotypic separation between mutant and wild-type neurons, with dose-dependent shifts upon β-amyloid oligomer exposure. These shifts correlated with increased neurofilament light chain (NfL) release—a translational biomarker of neurodegeneration—highlighting the assay's sensitivity to neurotoxic insults. OECD guidelines alignment in this case is particularly related to neurite morphology and network integrity. To complement neurotoxicity profiling, we developed neurite outgrowth and axonal regeneration assays using iPSC-derived dopaminergic and motor neurons (including neurodegenerative disease-relevant mutants). Neurodegeneration was induced via chemically defined triggers, with high-content imaging and viability measurements as key readouts. Regeneration was assessed using axotomy in microfluidic devices, enabling real-time tracking of axonal repair. These assays align with the neurite outgrowth and network formation endpoints in the OECD DNT in vitro battery and offer additional insight into functional recovery, complementing current regulatory tools. Together, these platforms provide scalable, multiparametric tools to assess compound-induced neurotoxicity and neurorepair potential. Their alignment with OECD test guidelines and guidance documents supports integration of neurotoxicity risk evaluation in early-phase drug development.

P199
Preclinical de-risking of cell therapy products
ABSTRACT #236

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Engineered T cell therapies such as Chimeric Antigen Receptor (CAR) T cells have emerged as promising therapies but also suffer from severe safety risks. The absence of relevant animal models underscores the need for robust in vitro safety evaluation. Human epidermal growth factor receptor 2 (HER2) is a well-established therapeutic target for various cancer types. To explore potential off-disease effects of HER2-targeting CAR-T cells, Retrogenix™ cell microarray screening was used to assess off-target binding against a

panel of >6,500 proteins. HER2 CAR-T cells were also co-cultured with primary or iPSC-derived cells to evaluate unwanted cytotoxicity against healthy cells. To de-risk potential insertional mutagenesis after stable CAR integration, HER2 CAR-T cells were assessed for uncontrolled, cytokine-independent growth, and vector copy number (VCN) was determined by droplet digital PCR. Finally, lipid nanoparticles (LNPs) were explored as a non-integrating delivery method for generating mRNA-encoding CAR-T cells. Although the Retrogenix™ screen identified only on-target and CAR-independent interactions with no off-target binding, HER2 CAR-T cells showed activation and cytotoxicity toward several primary cell types, likely due to on-target off-disease effects. A clear decrease in viability and cell numbers was observed when HER2 CAR-T cells were cultured without cytokines, supported by a low VCN of 3 (VCN <5 is recommended to minimize oncogenesis risk), indicating low transformation risk under tested conditions. LNP-mRNA CAR-T cells showed HER2 CAR expression in most T cells and cytotoxicity against a HER2-expressing tumor cell line. In conclusion, HER2 CAR-T cells were assessed in multiple in vitro safety assays. While no off-target binding or cytokine-independent growth was observed, cytotoxicity toward several primary cell types underscores the need for early safety evaluation. Furthermore, LNP-mRNA CAR-T cells demonstrated strong efficacy and represent a promising non-viral, non-integrating alternative for future cell therapy development.

P200
Skimune® a powerful human-centric platform for the identification of t cell engager immunotoxicity
ABSTRACT #308

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Background: Biologics and advanced cellular therapies demand robust safety assessment methodologies, highlighting the need for New Approach Methodologies (NAMs) that enable more predictive and human-relevant safety evaluation. Addressing this need, we aimed to develop and validate a human skin-based assay (Skimune®) for predicting adverse immune reactions to biologics, including T cell engagers, which are a novel class of bispecific biologics. One arm of the antibody binds CD3 on a T cell and the other arm(s) binds a specific target, however, these can be associated with immunotoxicity. Methods: Healthy skin tissue from the lower abdomen, along with 70ml of fresh blood were provided by healthy volunteers (n=10) recruited with informed consent via Local Ethics Committee approval from our local hospital. Blood samples were processed into purified Peripheral Blood Mononuclear Cell (PBMC) populations. Different T cell engagers were incubated with tissue samples and autologous PBMCs for 3 days. The tissues were then sectioned and stained for histopathological assessment of immunotoxicity using a clinical grading system of increasing damage severity (grades I-IV), including increasing dyskeratosis, vacuoles, sub-epidermal cleft formation, acantholysis and blistering. Culture supernatants were tested for multiplex cytokine assessment by MSD technology. Results: Using our Skimune® platform, grades of immunotoxicity were identified ranging from positive grades above background (grade I) to grade II and III depending on the engager and their different targets. Talquetamab targeting GPRC5D was used as a comparator, and this demonstrated immunotoxicity (grades II–IV) in ~65% of donors tested which is comparable with clinical trial results. TNF-α and IFN-γ were significantly elevated at the highest concentration of Talquetamab. Discussion and Conclusion: We have a novel platform which can be used to de-risk the development pipelines of T cell engagers, study the mechanism of action by cytokines and evaluation by developing specific skin pathology.

P201
Non human primate in vitro NAMs: a translational platform to complement human NAMs for efficient drug safety evaluation
ABSTRACT #363

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Background and Objectives: Recent regulatory initiatives, including the FDA Modernization Acts 2.0 and 3.0 and the FDA's April 2025 roadmap to reduce nonhuman primate (NHP) use in drug safety studies, have accelerated the adoption of human in vitro New Approach Methods (NAMs). However, a key limitation of human NAMs remains their restricted in vitro–to–in vivo translation, as unapproved compounds cannot be tested in human subjects before preclinical safety evaluation in animal models. With ExirBio, we propose a primate in vitro NAMs platform designed to complement human NAMs and improve translational decision-making in preclinical drug safety evaluation. Materials and Methods: The platform integrates induced pluripotent stem cell (iPSC)-derived systems, autologous immune components, and primary tissue–derived models from cynomolgus macaques (Macaca fascicularis), each linked to personalized genomic information. Together, these models enable human–NHP side-by-side in vitro testing both prior to and following NHP in vivo studies, reducing costs and providing additional mechanistic understandings. Results: Within ExirBio, three core systems are established: (1) NHP iPSC-derived neuronal models, including NGN2-induced and motor neurons exhibiting appropriate lineage markers and functional electrophysiological activity by multielectrode array (MEA) recordings; (2) NHP iPSC-based developmental toxicity assays incorporating AAV-based SALL4 reporters for evaluation of targeted protein degraders, enabling EC_{50} determination and dose–response profiling; and (3) primary tissue–derived cynomolgus gut organoids for mechanistic investigation of gastrointestinal (GI) toxicities observed in NHP in vivo studies. Discussion and Conclusion: Collectively, this primate in vitro NAMs platform supports Weight of Evidence (WoE)–based preclinical safety assessments, before initiating in vivo studies – all fully aligned with the FDA's 2025 NAMs roadmap. ExirBio's primate in vitro NAMs platform bridges human in vitro findings and NHP in vivo outcomes, enhancing predictive safety, guiding appropriate use of NHP studies, and advancing the 3Rs in drug development.

P202
Metabolic and cytotoxic readouts differentiate responses of 2D and 3D in vitro pancreatic ductal adenocarcinoma models to 5-fluorouracyl and paclitaxel
ABSTRACT #368

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Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal malignancies, with limited therapeutic response driven in part by stromal barriers and pronounced metabolic plasticity [1]. Because Conventional 2D cultures poorly capture tumor architecture and transport limitations, 3D spheroids are increasingly used to improve translational relevance in preclinical drug testing. Here, we compared the cytotoxic and bioenergetic effects of paclitaxel (PTX) and 5-fluorouracyl (5-FU) across two PDAC cell lines; PANC-1 (gemcitabine-resistant, mesenchymal-like) and BxPC-3 (gemcitabine-sensitive, epithelial-like). Additionally, the non-tumorigenic pancreatic ductal epithelial cell line hTERT-HPNE was used as a normal comparator [2]. Cells were cultured as both conventional 2D monolayers and scaffold-free 3D spheroids to model the transition from simplified to more tumor-like microenvironments. Drug sensitivity was quantified using the MTS assay to generate dose–response curves and estimate LD_{50} values in 2D versus 3D. Mitochondrial function was evaluated using the Seahorse XF Pro Mito Stress Test, reporting oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as indicators of oxidative phosphorylation and glycolysis-associated acidification, respectively [3]. Across models and cell lines, our ongoing analyses indicate culture-dependent differences in both drug sensitivity and mitochondrial metabolic responses to PTX and 5-FU. By integrating dose–response viability profiling with Seahorse bioenergetic readouts in 2D monolayers versus 3D spheroids, this work aims to clarify how culture geometry and PDAC phenotype shape cytotoxicity and metabolic adaptation. Upon completion, these data will support a more mechanistic comparison of PTX versus 5-FU in PDAC and strengthen the translational relevance of in vitro screening by pairing 3D models with mitochondrial metabolic profiling. Acknowledgment: This study was funded by the Croatian Science Foundation (project SPARK no. HRZZ-IP-2025-1762) and the European

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P203
Multicellular human intestinal new approach methodologies (NAM) with machine learning for diarrhea risk strattification across small molecules and antibody drug conjugates (ADC)
ABSTRACT #400

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Background: Drug-induced diarrhea is a frequent dose-limiting toxicity driven by multifactorial mechanisms involving epithelial barrier disruption and tissue stress. Animal models and standard 2D assays often fail to capture human intestinal physiology needed for early gastrointestinal risk decisions. To support lead optimization and candidate selection, we assessed whether a multicellular human intestinal barrier NAM integrated with machine learning (ML) can stratify diarrhea risk across small molecules and ADCs. Methods: A 3D human intestinal model (NAMkind™ Intestine) was established for ileum and colon using epithelial and stromal components that maintain polarity and tight-junction integrity. Under repeat-dose conditions, barrier function (TEER) and tissue health/redox state (ATP, Alamar Blue) were measured in a clinically anchored reference set of small-molecule drugs with known diarrhea risk (N = 48). ML models were trained using multivariate assay features and clinical toxicity labels. ADCs were evaluated using matched exposure schedules with intact ADC, antibody-only, and payload-only Results: The intestinal NAM was validated using the small-molecule reference set spanning multiple therapeutic classes. ML-integrated analysis of barrier function and tissue health endpoints demonstrated robust diarrhea risk stratification (>90% accuracy) and resolved differences within related chemical series. Within an NSAID series, diclofenac produced pronounced barrier disruption with a narrow margin of safety of 5 (MOS is the ratio TEER IC50:Plasma Cmax), whereas celecoxib exhibited minimal barrier impact and a 30-fold wider MOS of 150, consistent with clinical experience. Following small-molecule validation, the platform was extended to ADCs. Comparative profiling of intact ADCs alongside antibody-only and payload-only controls enabled separation of on-target, internalization-linked effects from off-target payload-driven liabilities, supporting preliminary risk ranking based on observed tissue responses. Conclusion: A multicellular human intestinal barrier NAM model integrated with ML provides a decision-oriented framework for early diarrhea risk stratification across different drug modalities, supporting mechanism-aware prioritization within non-animal safety assessment workflows.

P204
Multicellular human 3D liver new approach methodologies (NAM) for DILI risk assessment across small molecules and antibody drug conjugates
ABSTRACT #401

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Background: Drug-induced liver injury (DILI) remains a leading cause of clinical attrition and post-marketing withdrawal. Human-relevant NAMs capable of capturing cumulative functional impairment under repeat dosing are needed to support risk decisions across evolving drug modalities. To support lead-optimization and candidate-selection decisions, we assessed whether a multicellular human 3D liver spheroid NAM (NAMkind™ Liver) coupled with machine-learning (ML) calibration can stratify DILI risk across small molecules and ADCs. Methods: Multicellular 3D human liver spheroids composed of primary hepatocytes with non-parenchymal support cells were dosed repeatedly for up to 21 days. Tissue viability (ATP), membrane injury (LDH), and hepatocellular function (albumin) were quantified as a functional triad. Model performance was benchmarked using a clinically anchored reference set of 35 small-molecule drugs with known DILI outcomes, with additional within-class analogs included to support SAR-relevant decision making. ML calibration integrated multivariate

assay features with clinical labels to generate probabilistic risk scores. The platform was subsequently applied to an expanded set of 25 small molecules and to ADCs under matched repeat-dose conditions. Results: Using the initial reference set, the liver NAM reproducibly captured functional patterns associated with clinically relevant DILI risk and resolved differences among closely related analogs within shared chemotypes. High accuracy >90% was maintained when applied to 25 additional small molecules, indicating generalization beyond the original benchmark set. The platform was then extended to ADCs, which produced distinct concentration-dependent functional signatures across endpoints. These profiles enabled comparative assessment based on patterns of hepatocellular dysfunction rather than acute cytotoxicity alone. Model use-range checks and prediction-uncertainty estimates were implemented to identify atypical response profiles and prioritize confirmatory follow-up studies. Conclusion: A multicellular human 3D liver spheroid NAM integrated with ML provides a decision-oriented framework for early DILI risk stratification across small molecules and ADCs, supporting mechanism-aware prioritization in non-animal safety assessment.

P205
In vitro safety profiling of ADCs using an iPSC-derived human tissue screen to support FDA roadmap for biologics
ABSTRACT #410

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Antibody–drug conjugates (ADCs) and other targeted biologics continue to exhibit high attrition rates in clinical development, with dose-limiting and off-tumor toxicities contributing substantially to failure despite robust target engagement (1). Across phase I ADC trials, grade ≥3 treatment-related adverse events have been reported in up to 58% of patients (1), underscoring the need for predictive, human-relevant preclinical models. In alignment with the U.S. Food and Drug Administration's roadmap advocating mechanistically informed new approach methodologies (NAMs) for safety assessment (2), we developed a unified, fully human induced pluripotent stem cell (iPSC)-derived seven-tissue platform to identify on-target/off-tumor and off-target toxicity risks across therapeutic modalities. The platform comprises iPSC-derived cardiomyocytes, hepatocytes, melanocytes, motor neurons, vascular endothelial cells, alveolar epithelial cells, and intestinal epithelium—organ systems frequently implicated in biologic-associated toxicities. Cytotoxicity was quantified via real-time impedance (xCELLigence), and receptor expression was confirmed by high-content imaging. Cetuximab–MMAE was evaluated as a proof-of-concept ADC. The conjugate demonstrated tissue-specific, dose-dependent toxicity consistent with established clinical liabilities of cetuximab (3). iPSC-derived melanocytes exhibited the greatest sensitivity at 100 µg/mL with 56% toxicity and were the only tissue showing measurable toxicity (~10%) at clinically relevant concentrations (1 µg/mL), concordant with pharmacovigilance data identifying dermatologic adverse events as the most frequent and persistent cetuximab-associated toxicity (3). Tissues associated with toxicity at maximum ADC doses only; lung and liver at 48% and 28% respectively, aligns with EGFR expression. Tissues associated with limited clinical toxicity—including cardiac, central nervous system, and vascular systems—showed minimal in vitro responses, supporting predictive specificity. This fully human, multi-tissue iPSC platform provides a mechanistically grounded approach for early safety de-risking of ADCs and other biologics. By recapitulating the clinical toxicity fingerprint of cetuximab–MMAE, the model establishes proof-of-concept for translational safety profiling to inform candidate selection, optimize therapeutic design, and reduce downstream attrition.

P206
InVEST44™ is a cost-effective in vitro safety panel that identifies off-target effects in early stage drug discovery
ABSTRACT #415

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Drug development is costly and time-intensive, exceeding $172 million on average and surpassing $500 million when clinical trial failures are considered (Sertkaya 2024). As development can take up to 20 years, identifying safety liabilities early in discovery is essential to avoid investing resources in compounds that later fail during preclinical or clinical testing. Early in vitro safety panels provide a cost-effective strategy to detect toxicities and off-target effects before compounds progress to animal studies or human trials. The need for early safety assessment is reinforced by changing investment trends in the biopharmaceutical sector. Between 2006 and 2023, venture capital investment in early-stage drug discovery declined from 50% to 16%, while funding for later-stage development increased from 20% to 54%. This shift reflects increasing focus on expensive, high-risk clinical programs rather than early discovery. Improving early safety screening could reduce attrition by identifying compounds with unfavorable safety profiles before costly development stages. InVEST44™ is an in vitro safety pharmacology panel designed to detect off-target interactions of lead compounds in oncology and other therapeutic areas. The panel includes 44 high-risk targets based on Bowes et al. (2012), including GPCRs, transporters, ion channels, nuclear receptors, and enzymes. Assays include radioligand binding, fluorescence polarization, enzymatic activity, cell-based reporters, calcium mobilization, and electrophysiology. Twenty drug candidates from 17 failed oncology clinical trials (2001–2025) terminated due to severe adverse effects were screened at 10 µM. Seventeen compounds showed >50% activity on at least one target; eight interacted with four or more targets, and one affected 15 targets. These results demonstrate that in vitro safety panels such as InVEST44™ can identify clinically relevant off-target interactions before costly animal studies and unsuccessful clinical trials, supporting their growing inclusion in IND submissions (Scott 2022).

P207
A one health view on global outlook and future strategies for aninal testing replacement for vaccines: a report on the AFSA-IABS international conference
ABSTRACT #417

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Background & Objectives The global vaccine community is increasingly prioritizing the transition from animal-based quality control testing toward validated in vitro alternatives. This shift reflects a growing recognition that current products' life cycle management approaches, with good manufacturing practices, state of the art in process controls and scientific analytical tools can enhance the reliability, efficiency, and sustainability/ethical foundations of vaccine testing while maintaining or even improving, rigorous standards for safety and effectiveness. This also aligns closely with One Health principles and allows the vaccine field to support more sustainable practices while accelerating delivery of high-quality vaccines to populations worldwide. Materials & Methods To advance this goal, experts from both human and animal health sectors convened in Bangkok, Thailand, from 2–4 December 2025 for the conference "Animal Testing Replacement for Vaccines: A One Health View – Global Outlook and Future Strategy", organized by Animal-Free Safety Assessment and the International Alliance for Biological Standardization. It brought together regulators, scientists, manufacturers, and policy specialists from different regions to assess recent progress in developing NAMs for vaccine potency, safety and quality control. Results This poster describes the challenges that continue to slow global implementation but also successes achieved in replacing animals from the batch testing of vaccines. Barriers included fragmented regulatory expectations across countries, limited access to critical reagents and standardized reference materials, and uneven validation and acceptance pathways for alternative methods. In many regions, these differences create uncertainty for developers and manufacturers seeking to implement new approaches. Discussion & conclusion The conference concluded with a focused on three strategic priorities: 1. Strengthening of regulatory harmonization to support broader acceptance of validated in vitro methods. 2. Expanding practical reliance and

data-sharing mechanisms among institutions and regulatory authorities 3. Building sustainable global capacity to develop, validate, and routinely apply modern NAMs for vaccine quality control.

P335
Large particle imaging flow cytometry and plate-based sorting of intact 3D models to support reproducible new approach methodologies
ABSTRACT #443

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Human-relevant 3D in vitro models, including organoids and spheroids, are increasingly evaluated as New Approach Methodologies (NAMs) for toxicology and safety assessment. However, broader regulatory confidence in these systems requires analytical strategies that (i) characterize structural heterogeneity, (ii) define measurable and reproducible performance metrics, and (iii) support standardized downstream workflows. Conventional flow cytometry requires dissociation of multicellular structures, resulting in loss of spatial and morphological information intrinsic to intact 3D systems. Union Biometrica, Inc. instruments enable analysis and sorting of viable biological objects up to millimeter scale without dissociation. Integrated brightfield imaging (COPAS Vision™) further preserves structural context during analysis. HT-29 and HT-1080 are human cancer cell lines that can grow as ~200 micron spheroids. HT-29 and HT-1080 spheroid cultures (Corning Life Sciences) were subjected to analysis and sorting using the COPAS Vision equipped with a 1000 micron flow cell (Union Biometrica, Inc.). Cytometry: Analysis of HT-29 and HT-1080 spheroid cultures produced cytometric measurements of size (Time of Flight – TOF), optical density (Extinction), Forward Scatter, and fluorescence. GFP-positive HT-29 spheroids were selectively gated and dispensed singly into 96 well plates with 100% accuracy, demonstrating precise, automation-ready sorting of complex 3D cellular models. Profiler: Using the Profiler feature, up to 8,000 data points captured as each spheroid passed through the laser path were graphically and numerically displayed as a succession of peaks and valleys. Imaging: Captured brightfield images for each analyzed spheroid in flow. Image-linked cytometric records supported automated classification, real-time morphological QC, and traceability. These findings illustrate how large particle flow cytometry bridges a key gap in the NAM landscape by enabling scalable, automated, and data-rich processing of 3D biological models. This platform provides a technical foundation for broader deployment of 3D culture systems in research, screening, and regulatory-aligned applications.

P336
Current status and use of new approach methodologies in biological evaluation strategies
ABSTRACT #451

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Competent authorities worldwide are currently starting to accept new approach methodologies (NAMs) to validate the safety of pharmaceutical products, medical devices, and cosmetics. In 2025, the FDA's Modernization Act 3.0 (2025) reinforced the prioritization of NAMs and the reduction of animal use in drug development [1]. The UK devised a plan for phasing out in vivo eye irritation and skin sensitization testing by the end 2026 [2]. The newly released ISO 10993-1:2025 also encourages the 3R initiative for reducing, replacing and refining animal experimentation [3]. NAMs generate human-relevant data and have the potential to strengthen the decision-making for data poor constituents. Hence, NAMs, when combined with existing data are great assets for our regulatory toolkit to strengthen the scientific foundation of biological safety. However, no single NAM can completely reproduce an entire human system. Thus, the role of expert biological safety specialists and toxicologists in conducting gap analyses, suitability assessments, and interpreting results remains crucial. One important question that manufacturers and industry stakeholders need to answer is: How can you choose the

appropriate NAM? To answer this question, this poster will focus on NAMs used to evaluate toxicological endpoints (e.g. sensitization, irritation, cytotoxicity, and genotoxicity) in the medical device industry, and highlight the benefits of implementing them in your biological evaluation plans (BEPs). These NAMs include application of organs-on-a-chip, in silico modeling, read-across, Threshold of toxicological concern (TTC)-based screening, and systematic literature reviews to create robust and tailored risk assessments. Through a toxicological perspective, we show how NAMs can phase out "traditional" testing methods, and highlight the challenges that still persist. Importantly, we will discuss examples of how to overcome these challenges, to facilitate global regulatory compliance and support stakeholders' decision-making process. Adoption of these innovative methods could also improve the cost-effectiveness of regulatory testing while fulfilling 3R objectives.

P337
3D SPHEROID PERFORMANCE OF RAT AND HUMAN HEPATOCYTES COMPARING MEDIUM AND MICROPLATES
ABSTRACT #476

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Background: 3D hepatocyte spheroids are utilized due to their culture longevity and functionality as in vitro models for hepatotoxicity and pharmacokinetic studies. Spheroid viability and function can vary depending on their culture microenvironment. These studies tested: 1) the influence of two different culture media on pre-clinical rat hepatocyte (RH) spheroid morphology and performance and 2) compared morphology and performance of primary human hepatocyte (PHH) spheroids cultured in two types of ultra-low attachment microplates. Methods: Cryopreserved RHs were thawed, counted, and resuspended in TruVivo® Flex (TVF) or TruVivo® (TV) Plating Medium (PM). PHHs from normal and diseased livers were thawed, counted, and resuspended in Human Hepatocyte (HH) PM. Both species were seeded in Corning 96-well ultra-low attachment microplates. PHHs were also seeded in 96-well Nunclon™ Sphera™ U-shaped bottom microplates. Spheroids were cultured in TVF or TV Culture Medium (CM) (rat) or HHCM (human) for 21 days. Morphology, urea, and ATP levels were determined on days 7, 14, and 21. Human CYP3A4 activity was determined. Results: RHs formed spheroids by culture day 7 and were maintained for 21 days in both media. Urea function was similar between media throughout 21 days. ATP levels were higher in spheroids cultured in TVFCM versus TVCM except on day 21. PHHs formed spheroids in either microplate by day 7 and maintained morphology throughout 21 days. Urea levels and CYP3A4 activity were higher in spheroids cultured in the ultra-low attachment microplate compared to the U-shaped bottom microplate with the reverse being true for ATP levels. Conclusion: RHs formed spheroids and maintained urea function in TVFCM or TVCM demonstrating medium flexibility for this model. PHHs spheroid formation and function were supported by both U-shaped bottom and ultra-low attachment microplates. These results demonstrate cell, protocol, and medium reproducibility, which are all essential features for toxicity testing and translational applications.

P338
High-Definition Electrophysiological Assessment of FUJIFILM Human iPSC Neuronal Co-Cultures on CorePlate™ HD-MEAs[
ABSTRACT #478

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3Brain's CorePlate™ powered high-density microelectrode arrays (HD-MEA) enable non-invasive, high-resolution electrophysiological recordings of neuronal activity and in depth analysis of network dynamics. Traditional low density in vitro MEA recordings offer few data points resulting in low acuity and resolution to adequately capture network maturation, limiting translational potential of human based induced pluripotent stem cell (iPSC) neuronal models. To address this gap, we developed a protocol to culture FUJIFILM Cellular Dynamics human iPSC neurons and astrocytes on 3Brain's HD-

MEA (CorePlate™ 1W 30/60) to enable high resolution monitoring of the maturation of neuronal activity. Human isogenic iPSC-derived glutamatergic (iCell® Induced Excitatory Neurons), inhibitory (iCell® GABANeurons), and glial cells (iCell® Astrocytes 2.0) from FUJIFILM Cellular Dynamics were co-cultured on 3Brain's CorePlate™ HD-MEA to support long-term culture and robust network development. Recordings were performed longitudinally to monitor the maturation of synchronous bursting activity across the culture. Spontaneously active cultures displaying characteristic bursting patterns were observed after 25 DIV. HD-MEA recordings enabled robust quantification of activity changes over time, including an increase in firing rate, inter-spike interval (ISI) coefficient of variation, and peak-to-peak amplitude. Notably, there was a marked enhancement in both single-neuron bursting activity, shown by longer burst durations and a higher number of spikes per burst, and in network-level bursting properties. Within network bursts, network burst duration increased, the number of spikes per network burst increased and ISI decreased. The high spatiotemporal resolution of HD-MEA recordings facilitated detailed analyses of network connectivity, revealing a progressive increase in network complexity. These findings support the use of 3Brain's CorePlate™ enabled HD-MEA platforms and FUJIFILM Cellular Dynamics iPSC-derived neuron and glial co-cultures as a scalable, physiologically relevant tool for studying neural networks. The system is well-suited for applications in neuropharmacology, offering a translationally relevant alternative to animal models.

Organ-on-a-chip & Microphysiological Systems

P208
Evaluation of drug-induced liver toxicity of trovafloxacin and levofloxacin in a human microphysiological liver model
ABSTRACT #178

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Drug-induced liver injury (DILI) caused by approved pharmaceuticals represents a significant threat to patient safety and can result in drug withdrawal, regulatory setbacks, and substantial financial losses for the pharmaceutical industry. Current preclinical testing strategies, primarily based on two-dimensional cell cultures and animal models, often fail to reliably predict human-relevant liver toxicity. In this study, we leveraged a three-dimensional human liver-on-chip model to evaluate the hepatotoxicity of the withdrawn antibiotic trovafloxacin (TVX) and the structurally related, non-toxic levofloxacin (LVX) at clinically relevant concentrations. Liver-on-chip models were assembled using the Dynamic42 biochip, incorporating expandable liver sinusoidal endothelial cells and monocyte-derived macrophages in the vascular channel and HepaRG hepatocytes in the hepatic channel. Unidirectional perfusion, including drug treatment, was maintained for up to 7 days. Cell-specific morphology and viability were assessed using immunofluorescence and live-cell staining, while culture supernatants were analyzed for lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and pro-inflammatory cytokine release. Treatment with TVX elicited vascular and hepatocellular toxicity, accompanied by inflammatory cytokine secretion, whereas LVX caused no detectable tissue injury. Notably, the 3D liver-on-chip model was more sensitive than conventional 2D monocultures in detecting TVX-induced toxicity. Pro-inflammatory cytokine release was dependent on multicellular immune interactions, reflecting the idiosyncratic action of TVX. Mechanistic analyses further revealed hepatic glutathione depletion and mitochondrial reactive oxygen species formation as contributors to TVX toxicity. The model recapitulated TVX-induced injury that was not observed in murine studies lacking additional inflammatory stimulation. This proof-of-concept study demonstrates that the human liver-on-chip model can effectively evaluate hepatotoxicity, distinguish the DILI profile of TVX from the non-toxic LVX, and provide mechanistic insights into both immunomodulation and intrinsic toxicity. These findings highlight the potential of liver-on-chip systems as advanced in vitro platforms for

preclinical drug safety assessment, enabling early detection of adverse liver effects while reducing reliance on animal testing.

P209
Qualification of the PhysioMimix DILI assay in the 3RsC-FDA cross-platform DILI project
ABSTRACT #220

Emily Richardson[1], Justina Then[1], Isavella Georgiades[1], Ovidiu Novac[1], Kim Lachani[1], Tomasz Kostrzewski[1]
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The use of microphysiological systems (MPS) in safety testing is being increasingly examined by regulatory and standardization groups. Of these, liver MPS are the most advanced and well utilized across drug development, particularly for the investigation of drug-induced liver injury (DILI). However, there are no current regulatory accepted MPS solutions. For these reasons, the 3Rs Collaborative (3RsC) and FDA-CDER led a project to evaluate liver MPS for their ability to predict DILI. Eight blinded compounds were supplied to the nine commercial platform providers, the data generated independently analysed and subsequently disseminated to inform comparative assessments and insights into intra-platform variability by the regulators. The blinded compounds were tested in 6 concentrations in the PhysioMimix Liver-12 DILI assay, ranging from 0.05x to 10x Cmax. Media exchange, drug dosing and sample collection was completed every 2 days for 10 days, with 1 mL samples taken for analysis of ALT, AST, LDH, albumin, bile acid and cytokines. At days 4 and 10 of dosing, analysis of CYP3A4 metabolic activity was measured. At endpoint scaffolds were taken for brightfield analysis and lysed for further transcriptomic analysis. The PhysioMimix DILI assay identified four compounds as hepatotoxic and four as safe at the concentrations tested. Interestingly, three drugs were found to enhance the liver microtissues functionality at varying concentrations, particularly of albumin and bile acid production, as well as CYP3A4 activity. Whilst these agonistic activities were not necessarily hepatotoxic, it demonstrated the sensitivity of the assay to determine biological effects that may not otherwise be detected in other test systems. Together with other tested platforms, production of this dataset will further inform regulators and the wider field on the utility and robustness of liver MPS to predict complex hepatotoxicity and its utility within drug development pipelines.

P210
Scaling without compromise: liver MPS for lead optimization screens to investigative toxicology
ABSTRACT #225

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Drug-induced liver injury (DILI) remains the most common cause for acute liver failure in the USA and Europe and is a leading cause of attrition of compounds in drug development. Prediction of clinical safety is still a major hurdle in drug discovery and development, with many late-stage attrition events still occurring due to limited translatability of current in vitro or in vivo preclinical models. Microphysiological systems (MPS), otherwise known as organ-on-a-chip (OOC), provide a solution to this challenge by enabling the long-term culture and in-depth examination of highly human relevant liver microtissues. Here, we present two solutions based on their different contexts of use. Firstly, a higher throughput approach, the PhysioMimix® Multi-chip Liver-48 plate can be applied within the lead optimization process for screening of compounds, up to 288 samples (or chips) per run. Secondly, due to its enhanced tissue size and sampling volume, the Liver-12 plate can further characterize DILI events at a deeper level and their associated mechanisms of toxicity through a wide range of liver-specific endpoints. Using the PhysioMimix® Core System, primary human hepatocytes and Kupffer cells were cultured in 3D microtissues on engineered scaffolds in either the Liver-48 or Liver-12 plates under perfusion for eight days. For mechanistic studies, the Liver-12 microtissues were subjected to 10-day exposure to three tool compounds (diclofenac, troglitazone and pioglitazone). Liver-48 plate microtissues were able to successfully differentiate the DILI risks of the tool compounds, using liver functionality (albumin, urea, ALT, AST) and viability markers. By

assessing a range of test compounds recommended by the IQ MPS Consortium for DILI validation, the Liver-12 was able to correctly capture differing mechanisms of toxicity and mirror clinical outcomes. Oxidative stress, mitochondrial dysfunction, steatosis, dysregulation of bile acid synthesis or transport and inflammatory responses were investigated using a wide range of clinically relevant biomarkers.

P211
Integration of human hepatocytes and kupffer cells into an immune-competent liver organoids-on-a-chip model
ABSTRACT #228

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Background and Objectives. The liver plays a central role in drug metabolism, detoxification, and immune regulation, making it a primary target for drug-induced toxicity. Kupffer cells (KCs), the liver's resident macrophages, are key mediators of drug-induced liver injury (DILI) and contribute to conditions such as cholestasis, fibrosis, and viral hepatitis. However, the dynamic interactions between hepatocytes and KCs in response to toxicants remain poorly understood. Animal models used for liver toxicity assessment poorly represent the human immune system, while current in vitro liver models typically include only hepatocytes and lack immune components, limiting their predictive value in toxicology and immune research. To overcome these limitations, we developed a human hepatocyte–Kupffer cell organoid-on-a-chip (OoC) model, a microphysiological platform integrating key metabolic and immune components of the liver. Material and Methods. Functional hepatocyte-like cells (HLCs) and Kupffer-like cells (KLCs) were derived from induced pluripotent stem cells (iPSCs) and co-cultured as liver organoids. Immune competence of iPSC-derived KLCs was assessed relative to primary macrophages using lipopolysaccharide (LPS) stimulation as a positive control. HLCs metabolic function was evaluated by CYP3A4 expression and activity assays. After optimizing the culture conditions, organoids were cultured in a microfluidic chip to establish a two-cell OoC system and were used to assess (potential) hepatotoxicants. Results. The model demonstrated immune responsiveness, with secretion of proinflammatory cytokines TNF-α and IL-6 upon exposure to hepatotoxicants. In parallel, the system showed metabolic activity, with increased CYP3A4 expression (phase I metabolism) following toxicant treatment. Discussion and Conclusion. These preliminary results highlight the potential of this human-relevant hepatocyte–Kupffer cell OoC model as a robust platform for studying liver toxicity, immune responses, and inflammation-driven disease mechanisms, ultimately contributing to improved drug safety assessment and toxicology.

P212
Advancing NAMs with intestinal organoids: cross-species profiling of targeted protein degraders and hook effect dynamics
ABSTRACT #232

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Human-relevant models are central to the evolution of NAMs in toxicology. To support this transition, we established and characterized small intestinal organoids derived from mouse, dog, and human tissue. These organoids recapitulate key cellular populations from crypt to villus, offering a physiologically relevant platform for mechanistic studies and compound evaluation. In a pilot study, we conducted a cross-species comparison to evaluate bifunctional targeted protein degraders designed to reduce protein levels. Organoids grown in Matrigel domes were treated with selected compounds and analyzed using the

Jess system, an automated capillary-based Western blotting platform, following an optimized protocol for organoid-based protein quantification. Results revealed targeted protein decrease across all species, while the control inhibitor showed no change, supporting both the expected mechanism and assay specificity. Effects varied across conditions, with non-linear dose relationships consistent with ternary complex dynamics. Bifunctional degraders rely on forming a three-part complex between the target protein, an E3 ligase, and the degrader molecule to trigger ubiquitination. At high concentrations, the degrader can saturate both partners independently, preventing complex formation and reducing efficacy — a phenomenon known as the hook effect. To refine screening conditions, we transitioned to an array-based culture system (Corning® Elplasia® Microplates) for human intestinal organoids. This platform enables suspension growth within microcavities, improving standardization, homogeneity, and compound diffusion by eliminating Matrigel and ensuring uniform organoid size and arrangement. Using a nine-point dose-response design, we identified a clear hook effect for a target-selective degrader, highlighting the importance of dose optimization. Compounds lacking degradation capability or binding specificity did not reduce protein levels, confirming mechanistic specificity. Together, these studies demonstrate the utility of intestinal organoids combined with automated protein analysis in mechanistic toxicology. Integrating NAMs with advanced cellular models and analytical tools provides a scalable framework for cross-species evaluation and supports development of safer, targeted therapeutic strategies.

P213
A human liver-on-chip platform integrating HepaRG cells and LSECs to model hepatic function
ABSTRACT #238

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Drug-induced liver injury (DILI) remains a major cause of drug attrition and withdrawal, highlighting the need for predictive in vitro liver models. Conventional 2D hepatocyte cultures often fail to replicate the complex liver microenvironment and intercellular interactions critical for hepatic responses to xenobiotics. This study aimed to develop a microphysiological system (MPS) combining HepaRG hepatocytes and primary LSECs and to characterize its functionality relative to classical 2D cultures. The MPS chip comprised two channels, containing HepaRG™ cells or LSECs, separated by a communicating hydrogel (20 mg/mL alginate). In parallel, HepaRG cells were cultured under static conditions as monocultures or co-cultures with LSECs. Hepatocyte differentiation was assessed via HNF4α immunofluorescence and bile canaliculi formation using the fluorescent probe Fluobile™ 1. Albumin secretion was quantified by ELISA and visualized by immunofluorescence. Transcriptomic profiles were compared across static (mono- and co-cultures) and chip-based (mono- and co-cultures) conditions using Bulk RNA Barcoding sequencing (BRB-seq). Activities of nine major CYP450 enzymes were measured using Xenomix™ CYP substrates and LC-MS/MS. HNF4α and albumin labeling, together with quantitative albumin measurements, demonstrated high hepatocyte differentiation and functionality in the liver-on-chip model, supported by bile canaliculi formation. Albumin secretion increased over time, with greater levels in co-culture and in the chip versus static cultures. Transcriptomic analysis revealed upregulation of detoxification and fatty acid metabolism genes, and downregulation of immune response and hypoxia genes, compared to classical co-culture. CYP2A6 activity was higher in the liver-on-chip system for both mono- and co-cultures relative to traditional culture systems. We successfully developed a HepaRG-LSEC liver-on-chip model that demonstrates enhanced hepatocyte differentiation and functional albumin secretion. This platform provides a promising tool for studying hepatocyte function, intercellular crosstalk, and drug-induced liver injury. Incorporation of additional hepatic cell types could further improve physiological relevance and recapitulate the hepatic microenvironment more closely.

P214
Translational toxicology: adapting liver microphysiological system for cross-species evaluation of hepatotoxicity risk
ABSTRACT #242

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Drug-induced liver injury (DILI) remains a leading cause of drug candidate attrition during development, underscoring the need for improved translational models that can better predict human-specific toxicities. Traditional animal models, while informative for systemic toxicity, often fall short of accurately forecasting human liver responses due to inherent interspecies differences. To address this translational gap, we leveraged the PhysioMimix® Organ-on-Chip (OOC) Core System to develop a robust DILI assay incorporating primary hepatocytes from human, rat, and dog sources. Using the PhysioMimix Core System, hepatocytes from each species were cultured as 3D liver microtissues under dynamic perfusion in the Liver-12 plate for up to 14 days, maintaining stable, species-specific functionality (CYP activity, albumin, urea). A panel of six reference compounds - Troglitazone, Pioglitazone, Nefazodone, Buspirone, Tolcapone, and Entacapone - recommended by the IQ MPS Consortium, was applied across a seven-point dosing regimen over four days. This panel included both high-risk DILI agents and structural analogues with low human liability. Functional and clinical biomarkers (albumin, urea, LDH, ALT) were monitored to asses hepatocellular health and viability. The assay successfully captured known species-specific toxicities. Nefazodone and troglitazone (DILI Rank 8) induced hepatotoxicity in human and rat models, whereas dog hepatocytes showed reduced sensitivity. Conversely, buspirone and entacapone exhibited minimal toxicity in animal models, though subtle hepatotoxic signals were detected in the human MPS at higher entacapone concentrations, demonstrating its sensitivity to rare DILI events. Albumin consistently emerged as a sensitive cross-species biomarker, showing dose-dependent decreases aligned with known toxic profiles. These findings demonstrate the value of integrating human and animal liver MPS models to enhance cross-species interpretation and improve in vitro–in vivo extrapolation (IVIVE). The PhysioMimix system enables straightforward, parallel testing across species, supporting refined liver safety assessments, reducing animal use, and improving prediction of human hepatotoxicity risks early in the drug development pipeline.

P215
Impact of intestinal model complexity on deoxynivalenol-induced cytotoxicity: a comparative in vitro study
ABSTRACT #251

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In recent years, several scientific assessments from regulatory authorities and alert systems have identified mycotoxins as one of the most prevalent food and feed contaminants with significant health risks to humans. Among these, deoxynivalenol (DON), a type B trichothecene primarily produced by Fusarium species, has gained increasing attention due to its high prevalence, predominantly in cereal grains. In line with the current direction of modern toxicology, which emphasizes the use of human-relevant new approach methodologies (NAMs), the present study evaluated and compared the cytotoxic effects of DON exposure across different human intestinal cell culture models. Specifically, acute cytotoxicity after 48 hours of DON exposure was assessed in Caco-2/TC-7 human colon adenocarcinoma cells cultured as two-dimensional (2D) monolayers and three-dimensional (3D) spheroids, as well as in human induced pluripotent stem cell (hiPSC)-derived intestinal organoids. Based on the results, notable differences in half maximal inhibitory concentration (IC_{50}) values were

observed among the models. As expected, 2D Caco-2/TC-7 cells were the most sensitive to DON, with an IC_{50} of 6.50 µM. In contrast, no IC_{50} values were obtained for 3D cultures within the tested concentration range (2–128 µM); instead, viability decreased steadily to 60% at concentrations between 2–64 µM, reaching 40% only at the highest concentration tested (128 µM). Overall, our findings highlight the importance of evaluating mycotoxin cytotoxicity across multiple, complex culture models. The effects observed in 2D monolayers were not replicated in more complex 3D systems, suggesting that models with higher physiological relevance, possessing relevant enzymes and transporters involved in xenobiotic metabolism and excretion, may better reflect in vivo responses and reduce the risk of overestimating toxicity.
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P216
Modeling CAR-T cell migration and efficacy in a microphysiological system
ABSTRACT #253

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Microphysiological systems (MPS) offer a promising platform to evaluate the efficacy and safety of T-cell-based immunotherapies, such as chimeric antigen receptor (CAR) T-cell therapies, in vitro. This study establishes an MPS to assess the motility and migration of CAR-T cells between endothelial and epithelial compartments, simulating tissue homing. Induced pluripotent stem cell (iPSC)-derived endothelial cells, donor PBMC-derived T cells, and a transwell-based system were used to evaluate migration and cytotoxicity against CD19-expressing Nalm6 cells on-chip. CAR-T cells were engineered via CRISPR-Cas9 knock-in and expanded for functional testing. Migration was investigated under varying chemokine gradients, and flow cytometry and imaging quantified migration and activation. Cytotoxicity was evaluated by measuring the abundance of CD19 wild-type GFP (WT) and knockout RFP (KO) Nalm6 cells in the apical compartment. Key findings revealed that endothelial cells are essential for CAR-T cell migration, and chemokine gradients significantly influence motility. Notably, allogeneic CAR-T cells exhibited reduced migration compared to donor PBMC-derived T cells. The presence of Nalm6 cells did not alter CAR-T migration but confirmed robust killing, with selective depletion of CD19-expressing Nalm6 cells in the apical compartment. This work highlights MPS's potential to model immune cell migration and tissue-specific homing in preclinical CAR-T testing. The platform provides a precision medicine approach by incorporating autologous systems, reducing animal model dependency.
This project was funded by the European Union under Grant Agreement Nr. 101057438 (https://www.genetiga-horizon.eu/). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Health and Digital Executive Agency (HADEA). Neither the European Union nor the granting authority can be held responsible for them.

P217
Opportunities in toxicity testing of new drug modalities with 3D in vitro models
ABSTRACT #258

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Emerging drug modalities offer new opportunities to treat previously unmet diseases but also introduce complex, unpredictable side effects. Traditional nonclinical safety testing lacks translatability, driving the need for better methodologies. Human 3D in vitro models provide more physiologically relevant systems that may improve safety assessment by revealing modality-specific toxicities. The OrganoPlate® platform, a microphysiological system was used to generate organ models known to be affected by different drug modalities including small compounds, antibody drug conjugates

(ADCs), and AAVs (adeno-associated virus). The OrganoPlate comprises up to 96 individual chip, patterned underneath a 384-well microtiter plate. This innovative platform allows researchers to explore a wide range of flow and co-culture conditions, in a set-up that is compatible with standard laboratory equipment as microscopes and plate readers. The limited volumes required for the individual models allows the use of a range of starting materials, including primary cells, iPS (induced pluripotent stem) cells, and ASC (adult stem cell) derived organoids. We have developed several organ-on-a-chip models suitable for detecting different forms of toxicity. A neurite outgrowth model based on iPSC motor neurons extending their neurites into an extracellular matrix was leveraged to predict toxicity levels of ADCs. We were able to generate colon tubules using Caco-2 cell line, and ASC colon organoids. Their barrier integrity could be monitored through measuring the transepithelial barrier resistance, which reduced over time upon exposure to a panel of known gastrointestinal toxicants. Finally, we developed a liver MPS which exhibits unprecedented fidelity with the organization and interactions found within the native tissue by embedding primary or stem-cell derived liver cells in a hydrogel. The resulting culture includes polarized hepatocytes, functional vasculature with associated stellate cells and functional resident immune cells. This liver model was used to detect small molecule liver toxicity, as well as AAV transduction efficiency and toxicity.

P218
3D human intestinal organoids as a platform to assess compound-induced gastrointestinal toxicity
ABSTRACT #263

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Gastrointestinal (GI) toxicity remains a major barrier to the clinical translation of cancer therapies, often manifesting as diarrhea, mucosal ulceration, and intestinal inflammation. These adverse events, commonly associated with tyrosine kinase inhibitors (TKIs) and immunotherapies such as bispecific antibodies, are linked to epithelial barrier disruption and immune-mediated tissue damage. Given the intestine's central role in drug absorption and metabolism, early detection of compound-induced GI toxicity is critical in drug development. To address this, we employed 3D human induced pluripotent stem cell-derived intestinal organoids (HIOs) as a physiologically relevant preclinical model. We profiled the toxicity of clinically relevant chemotherapeutics—Afatinib, Irinotecan, and 5-Fluorouracil—using complementary viability assays and high-content imaging. For Afatinib, we introduced kinetic profiles aligned to clinical AUC and integrated pharmacokinetic data into mathematical models to estimate GI toxicity risk. Finally, we established a co-culture system of HIOs with peripheral blood mononuclear cells (PBMCs) to model immune-mediated epithelial injury and assess the GI liability of EpCAM-targeting bispecific antibodies. Our findings demonstrate that HIOs combined with kinetic modeling provide a scalable, sensitive platform for detecting epithelial damage, capturing both direct cytotoxic and immune-related effects. This organoid-based framework offers a powerful tool for early-stage screening, enabling prediction and mitigation of GI liabilities prior to clinical translation.

P219
Predicting gastrointestinal toxicity using the assay-ready, primary-derived OrganoReady® colon organoid model
ABSTRACT #286

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Gastrointestinal (GI) toxicity is among the most common adverse effect observed in clinical trials and after drug approval, often resulting in treatment delays, reduced patient quality of life, or even therapy discontinuation. Accurately predicting GI toxicity for novel drugs remains challenging, as many existing intestinal models fail to capture the full complexity of the gut epithelium. To address this limitation, we

developed the OrganoReady® Colon Organoid model, which employ automated seeding and tubule formation of primary-derived colon organoids. Our platform integrates 64 microfluidic chips within a single plate, each supporting fluid flow and providing access to both apical and basolateral compartments, thereby enabling high-throughput screening of small- and large-molecule therapeutics. Staining analyses confirmed effective polarization of the gut tubules and the presence of key differentiated colon cell types. Functional assays demonstrated that the OrganoReady Colon Organoid tubules recapitulate essential intestinal functions, including drug transport, metabolism, and barrier integrity. We exposed the OrganoReady Colon Organoid model with a representative panel of GI-toxicity-inducing compounds. After 72 hours, we assessed both viability and barrier integrity using Trans-Epithelial Electrical Resistance (TEER) measurements. The Colon Organoid tubules displayed greater sensitivity than conventional Caco-2 models. To evaluate reproducibility, we quantified intra- and inter-plate variability. Results showed minimal variation between chips within a single plate (Z' ≥ 0.5) and strong reproducibility between plates. Additionally, the model remained stable during shipment under controlled temperature conditions, allowing end users to perform large-scale compound screening following a short recovery period. Together, the OrganoReady Colon Organoid represents a robust and highly predictive platform for GI toxicity testing, offering multiple functional readouts to support safety-related decision-making. Furthermore, this platform lays the groundwork for future GI toxicity studies incorporating donor diversity.

P220
An air–liquid interface (ALI)-compatible, magnetically actuated platform for programmable cyclic stretch in human alveolar in vitro models
ABSTRACT #345

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Mechanical stretch is an essential physiological component of the alveolar microenvironment, and a number of stretch-enabled lung-on-chip systems and commercial platforms have been developed to reproduce breathing-like deformation. However, many of these systems rely on external pneumatic lines or custom chambers and are not readily compatible with routine insert-based air–liquid interface (ALI) culture workflows. To address this practical gap, we developed a compact, self-contained, magnetically actuated platform that directly integrates with standard culture inserts, enabling cyclic stretch under ALI conditions while minimizing handling complexity and potential contamination during long-term culture. The device employs a neodymium permanent magnet positioned above an elastic membrane cell culture insert and an electromagnetic coil beneath the culture plate, enabling programmable cyclic vertical deformation. Device performance was experimentally validated. Real-time imaging confirmed reproducible membrane displacement within the physiological range (approximately 0.2 Hz and 5–10% strain). Thermal measurements demonstrated stable culture medium temperature during actuation. To evaluate biological feasibility, alveolar type 2-like human pulmonary alveolar epithelial progenitor cells propagated with YAC (spell out) (Cite your BBRC paper) were cultured on collagen-coated microporous PDMS membranes integrated into the platform. Cells successfully attached to the membranes and formed confluent epithelial monolayers under culture conditions. Epithelial morphology remained stable during cyclic actuation, confirming compatibility between mechanical stimulation and epithelial growth. Together, these findings demonstrate both mechanical and biological feasibility of the system. This platform provides a practical foundation for incorporating cyclic stretch into human-relevant ALI alveolar models and supports future applications in respiratory mechanobiology and in vitro toxicology research.

P221
Elucidating gut-liver interactions in first pass metabolism of drugs using on-chip perfused and direct oxygenated MPS (BioStellar™ plate)
ABSTRACT #348

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Orally administered drugs undergo first-pass metabolism in the gut and liver before reaching systemic circulation, reducing effective drug exposure. However, the contribution of gut and liver crosstalk to first-pass metabolism remains poorly understood. MPS offers dynamic flow, oxygenation, and coculture of human-derived cells, enabling improved modeling of organ interactions. This study aims to establish a human-relevant gut-liver MPS coculture and to elucidate how organ interactions influence first-pass metabolism. A coculture model was constructed using iPS-derived intestinal epithelial cells and PXB-cells (humanized chimeric mouse hepatocytes) in BioStellar™ Plate [1, 2] with InnoCellTM oxygen permeable membrane. We evaluated the effects of perfusion, oxygen supply, and coculture on cell morphology, TEER, albumin secretion, and CYP activity. RNA-seq was used to identify transcriptional changes underlying organ interactions. Midazolam (MDZ) metabolism was assessed on both gut and liver compartments, and clearance values were integrated into PK model to predict human first-pass metabolism. Perfusion, oxygenation, and coculture jointly improved hepatic metabolic function, with the fully optimized condition showing the highest CYP activity [3]. RNA-seq highlighted arachidonic acid (ARA) and bile acid as a key mediator, enhancing CYP2D6 expression. MDZ studies demonstrated sequential hepatic metabolism and modest intestinal metabolism. After physiological scaling, extrapolation using coculture-derived clearance values showed close agreement with reported MDZ first-pass data in humans. The developed gut-liver MPS successfully reproduced organ-organ interactions and provided quantitative predictions of first-pass metabolism comparable to existing in vitro/in vivo platforms. The identification of ARA-mediated regulation of CYP2D6 highlights a previously unrecognized mechanism of gut–liver communication influencing drug metabolism. This simple setup offers a practical and mechanistic tool for studying inter-organ interactions and improving human PK prediction.

P223
Single cell printing enables adaptive tumor immunity interactions
ABSTRACT #365

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Background and Objectives The cellular heterogeneity, dynamics and spatial heterogeneity of tumor adaptive immunity pose great challenges to tumor-immune cell co-culture studies. Conventional large-cell-scale models often mask the intrinsic diversity of tumor and immune cells, missing the essential information for elucidating immunotherapy mechanisms, discovering rare cell subtypes, and screening cell surface receptors. A single-cell-scale interaction model has the potential to unravel the dynamic processes among specific cell subtypes, uncovering the differences in cell interaction and the resulting divergent outcomes. Material and Methods Here we adopted the alternative viscous-inertial force-based jetting (AVIFJ) to construct in vitro single-cell level immuno-oncology models (SLIMs) to reveal the dynamics and inhomogeneity of the interactions among DCs, T cells and mouse melanoma cells. Results The cells within this model successfully recapitulate key motility characteristics of adaptive immunity, including recognition, presentation, cytotoxicity, and immunosuppression. The dynamic results revealed a strong correlation between cell interaction and both motility and spatial distribution, specifically showing that the duration required for contact increased by 4–6 times when the distance exceeds 60 μm. Discussion and Conclusion Tracking and analysis of T cells further revealed the consistency between motility and biofunctions under the stimulation of different dendritic cell types, T cell subtypes, and stimulatory factors. The new SLIMs allow direct observation of immune cell interactions and their outcome, bridge cell motility and biofunctions, and further facilitate deeper analyses in the identification of

clinically relevant cell interaction processes and bridge different time and spatial scales.

P224
Microfluidic tumor-on-chip platforms for modeling tumor-immune cell interactions and patient-specific immunotherapy responses ex vivo
ABSTRACT #388

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Tumor-on-chip models represent a significant advance in oncology research, addressing critical gaps in traditional in vitro cancer studies. While murine models have been valuable, they often fail to accurately replicate human cancer biology, particularly regarding immunotherapy responses(1). Conventional in vitro studies typically lack the complexity of the human tumor microenvironment, three-dimensional structure, and dynamic interactions between tumor and immune response. Tumor-on-chip models offer a sophisticated alternative that can better recapitulate human cancer pathophysiology and physiological responses to cancer therapies. These systems are particularly relevant for heterogeneous, hard-to-treat cancers. Tumor-on-chip models aim to emulate the 3D architecture, cellular diversity, vascularization, dynamic endocrine and paracrine processes between tumor and immune cells, providing a more physiologically relevant platform for drug testing, elucidating mechanisms of action and understanding disease mechanisms. We developed various tumor-on-chip models using bespoke microfluidic immunocompetent platforms that integrate tumor tissues of different complexity ranging from cancer cell line spheroids, patient-derived microtumors or patient-derived tumor organoids (PDOs) within a hydrogel-based microenvironment. This setup facilitates a deeper understanding of cancer-immunity dynamics and the evaluation of therapeutic interventions, improving the discovery of new and improved advanced therapies. Here, we present concepts and findings from multiple studies utilizing distinct tumor-on-chip models for assessing drug efficacy and toxicity, including immunotherapies such as CAR-T cell treatments, immune checkpoint inhibitors and nano vaccines. These microphysiological tumor-on-chip platforms represent a significant advancement in cancer research. By closely simulating the crosstalk between human immune cells and the tumor microenvironment, these models increase the understanding of tumor biology, support the development of advanced treatment strategies and improve the predictive accuracy of preclinical testing not reliant on animal models.

P225
Amitraz induces cytotoxicity and functional alterations in neuronally differentiated SH-SY5Y spheroids
ABSTRACT #389

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In vitro cell culture models play a critical role in assessing the neurotoxicity of environmental contaminants, such as pesticides, by facilitating mechanistic studies and reduce reliance on animal models. Three-dimensional (3D) models of SH-SY5Y cells exhibit greater physiological relevance than two-dimensional (2D) monolayers. Nevertheless, the robust neuronal differentiation in 3D remains a methodological challenge. We have recently established and

optimized a neuronal differentiation protocol for SH-SY5Y spheroids, achieving a reproducible cholinergic (ChAT$^+$) phenotype within 22 days. The aim of the present study is to investigate the mechanisms of toxicity induced by amitraz (AMZ), a formamidine acaricide, which is sometimes detected in honey above regulatory limits. The cytotoxicity of AMZ was measured by quantifying ATP levels after 24 h exposure to increasing concentrations of AMZ, and the IC50 was determined. The cell death processes (apoptosis/necrosis) were determined by means of flow cytometry with annexin V conjugated with propidium iodide (vital DNA dye). And, neuronal functionality and toxin-induced dysfunction (neurotoxicity) was determined by means a Fluor-4 calcium imagen kit and the expression of neuronal markers (ChAT+ and synaptophysin), using immunofluorescence and confocal microscopy. The results demonstrated an IC50 value of 378 µM for AMZ. Exposure to this concentration induced late apoptosis and significantly impairment of neuronal functionality, as evidenced by altered calcium signaling related to changes in ChAT and synaptophysin immunofluorescence. In conclusion, AMZ was observed to induce neuronal dysfunction in differentiated SH-SY5Y spheroids. Consequently, this model provides a robust platform for the determination of neurotoxic effects induced by environmental contaminants such as AMZ. Acknowledgments: This research was funded by MICIU /AEI /10.13039/501100011033 / FEDER, UE through the project PID2024-160527OB-I00. F. F.C. thanks to the scholarship "Santiago Grisolía (CIGRIS/2021/073)". V.Z. acknowledges support from the Programa Beatriz Galindo fellowship (BG23/00107) of the Spanish Ministry of Universities and from Generalitat Valenciana (CIGE/2024/133).

P226
Engineering of cancer microenvironments with embedded interconnected microcapillary networks via in vitro magnetic assembly of endothelial-cell ‘seeds’. ABSTRACT #390

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Despite significant developments in endothelial-cell (EC) manipulation techniques, a proper in vitro model of a functional microvasculature with controlled local interconnectivity under well-defined global architecture is still lacking. Here, we report the generation of such controlled multi-scale vascular networks via manipulation of tens of sprouting EC ‘seeds’ (1). We exploit magnetic patterning to assemble EC-coated superparamagnetic microbeads into ordered arrays and establish effective growth rules governing the development of interconnectivity and directionality of the networks depending on the applied seed- seed spacing. We demonstrated that the spatial organization of the microvasculature can be controlled at both microscopic and mesoscopic level via directed-assembly of EC-microcarriers. We use our system to establish a critical seed-seed spacing below which the neighboring microvasculatures become interconnected, and above which they remain disconnected, even at late times of culture. In the latter case, they can be treated as practically independent which allows systematic monitoring of multiple EC- seeds developing in nearly identical conditions. We show that EC-seed-based approach offers a range of advantages over conventional EC-manipulation techniques including: (i) expedited sprouting, (ii) spatial control over interconnections, (iii) reduction in cell consumption by >100x, and (iv) native high-throughput format. We demonstrated that the microvascular arrays co-cultured, e.g., with cancer cells, can efficiently serve as a high-throughput platform for functional high-content screening of various anti-angiogenic compounds in full 3D microenvironment. Moreover, our model allows to faithfully mimic various cancer microenviroments, and in particular study differences in capillary networks morphology depending on the tumor type, and its pro or antiangiogenic properties. Overall, we propose a uniquely precise and standardized vascular-microtissue engineering tool with applications, e.g., in angiogenesis research, high-throughput drug testing including personalized therapies, and with possible extension to immune microenvironments and organ-on-chip approaches. Research supported by grant no. 2022/45/B/ST8/03675 from Polish National Science Center.

P227
Impact of intestinal model complexity on deoxynivalenol-induced cytotoxicity: a comparative in vitro study
ABSTRACT #393

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In recent years, several scientific assessments from regulatory authorities and alert systems have identified mycotoxins as one of the most prevalent food and feed contaminants with significant health risks to humans. Among these, deoxynivalenol (DON), a type B richothecene primarily produced by Fusarium species, has gained increasing attention due to its high prevalence, predominantly in cereal grains. In line with the current direction of modern toxicology, which emphasizes the use of humanrelevant new approach methodologies (NAMs), the present study evaluated and compared the cytotoxic effects of DON exposure across different human intestinal cell culture models. Specifically, acute cytotoxicity after 48 hours of DON exposure was assessed in Caco-2/TC-7 human colon adenocarcinoma cells cultured as two-dimensional (2D) monolayers and three-dimensional (3D) spheroids, as well as in human induced pluripotent stem cell (hiPSC)-derived intestinal organoids. Based on the results, notable differences in half maximal inhibitory concentration (IC_{50}) values were observed among the models. As expected, 2D Caco-2/TC-7 cells were the most sensitive to DON, with an IC_{50} of 6.50 µM. In contrast, no IC_{50} values were obtained for 3D cultures within the tested concentration range (2–128 µM); instead, viability decreased steadily to 60% at concentrations between 2–64 µM, reaching 40% only at the highest concentration tested (128 µM). Overall, our findings highlight the importance of evaluating mycotoxin cytotoxicity across multiple, complex culture models. The effects observed in 2D monolayers were not replicated in more complex 3D systems, suggesting that models with higher physiological relevance, possessing relevant enzymes and transporters involved in xenobiotic metabolism and excretion, may better reflect in vivo responses and reduce the risk of overestimating toxicity. Acknowledgments: This research was funded by MICIU /AEI /10.13039/501100011033 / FEDER, UE through the project PID2024-160527. Martina Sánchez acknowledges the Generalitat Valenciana for the Santiago Grisolia Fellowship (CIGRIS/2023/071).

P339
Human iPSC-Derived NAMs for Safety Assessment: From Early Hazard Identification to Mechanistically Informed, Multicellular Models
ABSTRACT #467

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New Approach Methodologies (NAMs) are advancing safety assessment by enabling earlier, more human-relevant detection of toxicological liabilities. Human induced pluripotent stem cell (iPSC)-derived tissue models are increasingly applied across hazard identification, toxicity testing, and safety pharmacology to provide functional, mechanistically informative readouts that extend beyond traditional endpoint-based assays. Here, we present data demonstrating the utility of iPSC-derived cardiomyocytes, neurons, and hepatocytes cells in detecting compound-induced liabilities across cardiac, neural, and hepatic systems. In monoculture formats, these models support early detection of functional perturbations—including electrophysiological changes in cardiomyocytes and alterations in neuronal network activity—prior to the onset of typical terminal endpoints. These early signals enable more informed decision-making and facilitate mechanistic investigations that can guide medicinal chemistry efforts toward safer and more effective compounds. Complex culture systems expand the NAM approach to further

enhance physiological relevance across inter-linked physiological system. Here we present data on the impact of increasing biological complexity through multicellular systems of iPSC-derived diverse cell types including cardiac fibroblasts and endothelial cells; astrocytes, diverse neuronal subtypes and microglia; hepatocytes, macrophage (Kupffer-like cells) and hepatic stellate cells. Further, we will present data on a complete IPSC-derived blood brain barrier with demonstrated clinical translation and relevance. Across these systems, we demonstrate that increasing cellular complexity can refine sensitivity and provide deeper mechanistic insight, supporting a transition from reductionist assays to more predictive, human-relevant models. Collectively, these findings illustrate how iPSC-based NAMs can bridge early discovery and safety pharmacology, aligning with evolving regulatory and scientific priorities to improve translational predictivity and reduce late-stage attrition in drug development.

P340
A Versatile Microfluidic Device for High-throughput Combinatorial Drug Screening
ABSTRACT #481

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Traditional in vitro drug testing relies on multi-well plates, where precise drug concentrations must be manually pipetted into each well. This process becomes increasingly laborious, time-consuming, and error-prone, particularly for diseases requiring combination drug therapies such as in cancer chemotherapy. While liquid handling systems offer automation, they are costly, and microfluidic chips, though promising, often require complex external pumping systems and are challenging to operate. In this study, we developed a high-throughput microfluidic gradient generator for combinatorial drug testing. The device was designed using extensive simulation approach. The device is capable of creating combinations of two or three drugs in different ratios without manual intervention. The device tested for drug combinations as (Doxorubicin +5-Fluorouracil) on MCF-7 cells, and the result was validated using conventional MTT assay. This platform offers an affordable, scalable, and user-friendly solution for in vitro drug testing, particularly in the context of combination therapies, where multiple drugs must be screened simultaneously.

P341
From Action Potentials to Organoids: A Unified Optical Platform for Multi-Modality Safety Pharmacology in Human iPSC Models
ABSTRACT #488

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Safety pharmacology increasingly demands human-relevant functional data spanning multiple organ systems and tissue architectures, yet most microphysiological platforms are optimized for a single modality or format. Here, we demonstrate a high-throughput workflow capturing voltage, calcium, and contractility readouts across 2D monolayers and 3D engineered tissues using a single optical instrument. In hiPSC-cardiomyocyte monolayers, fluorescent voltage imaging resolved action potential dynamics with sufficient temporal fidelity to derive reproducible concentration-response relationships for reference ion channel blockers. Screening flecainide, amiodarone, and quinidine across an 8-point half-log dose curve (0.05 to 100 µM), four-parameter logistic fits of fluorescent amplitudes yielded sigmoidal relationships (all $R^2 > 0.95$) with IC50 values of 4.96, 0.84, and 0.41 µM, respectively. Additional waveform parameters, including rise and decay rates, were similarly quantified. In 3D engineered heart tissues (EHTs) cast in 96-well format, contractility readouts enabled efficient profiling of cardiac myosin modulators and L-type calcium channel blockade within a single plate (doses up to 5 µM). Mavacamten produced concentration-dependent twitch-force reduction (~85% inhibition at 5 µM), verapamil abolished spontaneous contractility by 5 µM, and omecamtiv mecarbil elicited >5-fold increases in twitch force, yielding mechanism-consistent functional signatures

within a single screen. 96-well 3D engineered muscle tissue (EMT) culture enables high-throughput AAV capsid evaluation, with longitudinal parallel quantification of transgene expression and contractility across MOIs. Among 10 myotropic capsids screened, the most efficiently expressed reporters induced dose-dependent functional impairment at elevated MOIs, including contractile force reduction to ~25% of controls and loss of calcium signaling. Finally, the same instrument extends to 384-well CNS-3D brain organoids, supporting acute and repeat-dose neuroactivity profiling. Calcium imaging captured mechanism-consistent responses to rotenone, 4-AP, MK-801, and muscimol across seven-point half-log dilutions, with reversible muscimol responses across repeated dose-washout cycles demonstrating longitudinal capability.

Other

P228
Convergence science: the 3Rs versus the NAMs
ABSTRACT #1

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The 3Rs and the NAMs are not new concepts. Although they should be known, there are still ambiguities about the respective paradigms. Those concepts are misunderstood by the civil society. As a result, one can observe more opposition and trench warfare than collaborative willingness to deliver an overall reduction of animal use. How to accelerate the transformation of concepts into reality? A change of mindset is required to foster development and convergence of technologies to address scientific questions. Convergence science and transdisplinarity encompass the juncture between diverse expertise, stakeholders, disciplines towards a more comprehensive integration. Working on the interface of disciplines accelerate technology development.

P229
Investigation of the effects of reduced gravity on liver using new approach methodologies
ABSTRACT #5

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Reduced gravity not only is a condition inherent to space, but also has a number of medical applications on Earth. However, the outcome of reduced gravity on metabolically highly active organs has been poorly documented thus far. Among those is the liver, which after all serves as the poison control center of the human body, hence being a frequent target of adversity. Nevertheless, a number of effects of reduced gravity on the liver have been observed in astronauts as well as in experimental animals during actual spaceflight and upon simulation of reduced gravity on Earth. These include changes in hepatic lipid and carbohydrate metabolism leading to early metabolic dysfunction-associated fatty liver disease. The mechanistic basis of these effects, however, remains elusive. This actually defines the scope of the present work. Both spheroid cultures of human liver cells and zebrafish larvae were subjected to reduced gravity using a random positioning machine. RNA sequencing was performed for holistic investigation of the impact of reduced gravity on the liver followed by validation at the protein level using immunoblot analysis. Focus was hereby put on effects on signaling pathways, biotransformation enzymes and drug transporters, most that were studied further at the functional level by means of various assays. These unprecedented studies not only shed light onto the mechanisms underlying the outcome of reduced gravity on the liver, but also showcase the use of new approach methodologies in emerging biomedical research disciplines.

P230
Multi-level characterization of liver-based in vitro models and their application to food-derived chemicals
ABSTRACT #13

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The shift toward human-relevant and mechanistically informed safety testing is essential for modernizing food risk assessment and reducing reliance on animal models. New approach methodologies (NAM), including in vitro methods, offer a promising path forward, yet their adoption in food safety remains limited compared to other regulatory sectors. Developing NAMs depend on cost-effective, standardized and human-relevant in vitro cell culture systems able to capture specific mechanisms relevant to hepatotoxicity. In fact, mildly complex in vitro systems are strongly preferred by industrial end-users. In this study, we aimed to identify the most physiologically-relevant in vitro liver model by performing a comparative characterization of four human liver-derived monolayer cultures: C3A, a widely used epithelial-like hepatoma HepG2 subclone; HepaRG®, a bipotent and metabolically competent cell line; HepaSH®, a newly developed cell culture derived from primary human hepatocytes engrafted in chimeric mice; and Upcyte® hepatocytes, genetically modified primary-like cells. A multi-level characterization under constitutive conditions was performed, comprising transcriptional profiling using RNA sequencing, semiquantitative evaluation of phase I and II metabolizing enzymes, qualitative assessment of transporter expression, and analysis of key liver-specific functional endpoints. Moreover, the models were tested for their ability to capture key events associated to cholestasis (e.g. bile acid transport, canalicular dynamics), steatosis (e.g. lipid accumulation, fatty acid metabolism), and stress responses (oxidative stress, endoplasmic reticulum stress, inflammation, apoptosis). Food related chemicals suspected of causing liver injury, specifically perfluorooctanoic acid, tetrabromobisphenol A, and aminomethylphosphonic acid, were then tested in the best performing model. Our work contributes to the development of a robust and human-relevant NAM for regulatory food safety assessment, emphasizing the importance of mechanistically anchored and standardized methodologies.

P231
Overcoming systemic barriers toward animal-free research through collaboration and critical reflection
ABSTRACT #64

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The transition toward animal-free research is gaining momentum, supported by advancements in Non Animal Methods (NAMs) and growing regulatory acceptance. In parallel, new approaches are emerging that address not just technical challenges, but also the systemic and ethical dimensions that often hinder broader adoption. Recent developments in the Netherlands illustrate how these deeper barriers can be actively and constructively tackled. A key milestone is the Policy Advice from the NCad (Netherlands National Committee for the protection of animals used for scientific purposes) released in 2026, which concludes that many barriers stem from deeply held values and assumptions. Meaningful progress requires making these explicit and subjecting them to critical reflection. Long-term change relies not only on continued innovation, but also on cultivating a culture integrating ethical reflection and societal engagement. The advice recommends a dual strategy: a long-term vision with structural policy measures to address systemic challenges, alongside realistic short-term goals that are regularly reassessed. The presentation will also highlight the role of the Dutch Centre for Animal-Free Biomedical Translation (Ombion CPBT) as a national and international driver of change supported by the Dutch Government and its Ministry of Agriculture. The CPBT promotes collaboration among key stakeholders – including researchers, industry, and policymakers. Through concrete examples, we will show how building trust and fostering alignment between these actors is essential for breaking through entrenched barriers. Taken together, the NCad Policy Advice and the work of the CPBT show that lasting progress in animal-free research depends not only on technological advancement but on a broader paradigm shift—one that includes ethical awareness, inter- and transdisciplinary cooperation, and long-term commitment. In addition to this, a government that takes a strategic leadership role is a prerequisite. This presentation offers practical insights aimed at supporting and advancing this essential

transition through shared learning and dialogue.

P232
Challenges and opportunities in evaluation of in vivo and in vitro irritation
ABSTRACT #69

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The evaluation of irritation is an essential part of the biological risk assessment of medical devices, and this is frequently addressed through in vivo biological testing. The in vitro reconstructed human epidermis (RhE) assay has already undergone rigorous test method validation; however, dual data demonstrating concordance between the in vivo and in vitro testing outcomes is desired. The intracutaneous rabbit test was performed on a device categorised as External Communicating Device, Indirect Blood Path, Long-Term contact duration as part of the biological safety evaluation. Irritation potential was observed in the non-polar extract. Potential causes of irritation were investigated through analysis of the biological testing outcomes, the extractables profile, and the physicochemical properties of the materials of construction and related compounds. Two potential culprits were identified – sterilization and solvent interference, i.e., inherent irritation potential from the non-polar solvent. The outcome of the investigation illustrated that solvent interference was a confounding factor in the interpretation of the in vivo testing results. The in vitro reconstructed human epidermis (RhE) assay was conducted to further evaluate the irritation potential. None was observed in any of the extracts, and interpretation of the results in the in vitro assay was not confounded by solvent interference. This case study highlights complexities in the evaluation of in vivo irritation testing outcomes (e.g. solvent interference) and the importance of understanding underlying reasons for non-concordant dual data. Evaluation of concordance between dual data across the spectrum of irritation, from non-irritating to irritating, is essential. Therefore, it is proposed to bring various stakeholders together to identify devices with testing outcomes representative of the full spectrum of irritation, recognising that marked devices/devices under development are proprietary information, requiring anonymization throughout the process.

P234
Development of an in vitro model incorporating adverse outcome pathways to assess aerosol effects on cellular mechanisms related to chronic obstructive pulmonary disease
ABSTRACT #75

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Chronic Obstructive Pulmonary Disease (COPD) is a collection of lung conditions including emphysema and chronic bronchitis. Chronic exposure to cigarette smoke and certain environmental pollutants can drive oxidative stress and inflammation in the lung, potentially leading to COPD. Adverse Outcome Pathways have mapped molecular initiating events and subsequent key events that lead to decreased lung function, a key cause of airway obstruction seen in COPD. These events can be modelled in vitro using 3D cultured human lung tissues. One benefit of using such 3D lung tissues is that they provide an air-liquid interface amenable to direct exposure to gasses, volatile substances and cigarette smoke. In this development study, MucilAir tissues were exposed to varying concentrations of both cigarette smoke and aerosols from next generation products (NGP), using a Vitrocell smoking robot and exposure modules. Three replicate tissues were used for each product, and deposited nicotine was measured as a marker of exposure. Positive controls were also included. Biomarkers of COPD were measured, including those involved in oxidative stress, cilia formation, number and beat frequency, altered fluid uptake, mucus viscosity and impaired mucociliary clearance. The study demonstrated that dose related increases in nicotine deposition correlated with increasing cigarette smoke or NGP aerosol concentration. Cytotoxicity, as measured by WST-8 was < 50% at the highest concentrations tested. Dose related increases in oxidative stress markers were observed following exposure to cigarette smoke and NGP aerosols. However, the NGP

aerosols induced responses only when tested to significantly higher aerosol concentrations for longer periods, as compared to cigarette smoke. A viable in vitro model for the assessment of an aerosol's propensity to cause oxidative stress and impaired mucociliary clearance relevant to COPD has been developed that utilises biologically relevant endpoints. Further products and controls should be tested to assess reproducibility and refine this model.

P235
From one lung to many tests: A NAM-compliant ex vivo lung slice model for inhalation toxicity assessment
ABSTRACT #77

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Background: The development and validation of human-relevant in vitro and ex vivo systems is a key priority within the paradigm of Novel Alternative Methodologies (NAMs). Precision-cut lung slices (PCLS), which preserve the multicellular architecture and functionality of the lung, provide an ethically acceptable and translationally relevant platform for testing inhalation toxicants. Objectives: This project presents a 3R-compliant strategy for generating multiple viable murine lung slices from a single animal, enabling a high-throughput approach to inhalation toxicity testing while minimizing animal use. Furthermore, the method is being extended to human lung tissue obtained from surgical biopsies, aiming to strengthen cross-species comparison and regulatory relevance. Material and Methods: Healthy C57BL/6 mouse lungs will be inflated with low-melting agarose and sectioned using a vibratome to obtain 250–400 µm slices. These will be cultured on transwell inserts and exposed to cigarette smoke, e-cigarette aerosol, or heated tobacco product emissions under air-liquid interface (ALI) conditions. Cell viability, inflammatory cytokine release, oxidative stress markers, and gene expression will be assessed using ELISA, RT-qPCR, microscopy, and immunofluorescence. A comparable workflow is under validation for human lung slices (hPCLS). Results: From each mouse, up to 20 viable lung slices can be consistently obtained and maintained in culture for up to 48 hours. The model supports multiple parallel experimental conditions per donor and demonstrates sensitivity to aerosol exposure with dose- and time-dependent responses. Initial feasibility data confirm that human lung slices can also be processed and maintained ex vivo. Discussion and Conclusion: This NAM-based ex vivo platform reduces animal usage while enabling multi-endpoint inhalation toxicology assessment. Its extension to human tissue enhances physiological and regulatory relevance. The system is particularly suitable for comparative testing of next-generation nicotine delivery systems and conventional tobacco products, supporting the refinement of safety assessments in line with the 3Rs and OECD testing strategies.

P237
Methods to assess delivered dose in air-liquid interface exposure systems.
ABSTRACT #80

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Tobias Krebs, Marco Hebestreit, Christoph Schlager, Paul Schumacher Vitrocell Systems GmbH, Waldkirch, Germany Exposure of cell cultures from the respiratory tract to gases, complex mixtures, and particles is most physiologically relevant when performed at the air–liquid interface (ALI). In this configuration, the apical surface of the cells remains uncovered by culture medium, closely replicating the conditions encountered in the human respiratory tract during inhalation. The gas, semi-volatile, and particulate phases of the test atmosphere can thus interact directly with the cell surface, without interference from the culture media. In contrast to submerged exposure, ALI also facilitates the implementation of robust dosimetry approaches, enabling accurate quantification of the delivered dose and its correlation to biological outcomes. Dosimetry can be assessed at two principal levels: (i) characterization of the test atmosphere, and (ii) determination of deposition at the cellular surface. Characterization of the test atmosphere involves measurement of gas-phase constituents, either comprehensively or focusing on selected key analytes using FTIR or TOF-MS techniques. For the particulate phase, parameters such as particle

concentration and size distribution are commonly determined using established aerosol measurement techniques like photometers and SMPS. Measurement of particle deposition at the cellular level requires dedicated tools capable of quantifying the actual dose delivered to the cell surface. Methods include, quartz crystal microbalances (QCMs) for in-line mass measurement, a novel lock-in thermography for thermal detection of deposited particles, and the use of transmission electron microscopy (TEM) grids to assess particle distribution and morphology. Furthermore, techniques for capturing and quantifying deposited material in liquids are available and may be applied depending on the aerosol properties. The selection of dosimetry methods should be guided by the specific objectives of the study, the technical capabilities of the laboratory, and the configuration of the exposure system employed.

P238
Enniatin B1 risk assessment in a new approach methodologies (NAMs) perspective
ABSTRACT #108

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Background: The emerging mycotoxin Enniatin B1 (EnnB1), among the 28 other enniatin analogs, is one of the most widespread Fusarium produced microfungi. It is present worldwide in cereals and cereal-based products that represent the main dietary source for humans. However, these emerging mycotoxins are not regulated due to the lack of toxicokinetic (TK) and toxicodynamic (TD) data that make it difficult to appropriately assess their risk. The aim of this study is to contribute, in a NAMs perspective i) to integrate EnnB1 in vitro TK parameters in a minimal Physiologically Based Pharmacokinetic (PBK) model ii) and to assess its hepatotoxicity in vitro. Methods: TK parameters as hepatic clearance and fraction unbound to plasma were determined on human and rat in vitro models. In silico predictions on physicochemical properties, were used to develop a PBK model when experimental determinations were not available. The hepatotoxicity was determined using an omics approach after a subacute exposure of hepatocytes. Results: Interspecies differences in TK parameters were observed, particularly the hepatic clearance higher in rats. The fraction unbound to plasma was low and slightly different between the two species. The in vitro TK parameters were introduced in the PBK model and have improved the predictions. Afterwards, the validation of the developed rat PBK model was performed using observed data in rats after an intravenous administration. The extrapolated model to humans allowed to determine the maximum concentration in the liver. Moreover, the hepatotoxicity was assessed based on an omics approach to determine the most sensitive mechanisms and a Benchmark concentration on in vitro liver models. Conclusions: The PBK model in humans represents a fundamental piece for EnnB1 risk assessment. Indeed, the EnnB1 predicted concentrations in liver could be compared to points of departure of hepatotoxicity, determined by the omics approach, to assess the risk in humans.

P239
Mechanistic-based multi-omics approach to investigate the hepatotoxicity of nanomaterials: a case study on synthetic amorphous silica used as food additive
ABSTRACT #114

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Synthetic amorphous silica (SAS, E551) is widely used in food industry as an anti-caking agent, thickener and flavour carrier. Despite its broad use, uncertainties remain in assessing the associated health risks of the SAS nanometric fraction due to limited toxicological data. Therefore, the integration of New Approach Methodologies (NAMs) is promising to refine risk assessment. In this study, the potential hepatotoxic effects of SAS were investigated in using multi-omics including proteomics and metabolomics in human HepaRG liver cells. HepaRG cells were exposed for 24 hours to four different E551 materials (NM200, NM203, NM112, NM113) as well as to the Sicastar model colloidal silica (20 nm) at 10, 50, and 100 µg/mL. Standard toxicological endpoints (cell viability, oxidative stress, inflammation) were measured, alongside proteomics, metabolomics and phosphoproteomics analyses. Additionally, protein stability using MG132, a proteasome inhibitor, was addressed for NM200/NM203. While no major cytotoxicity was detected, multi-omics revealed that SAS can affect various cell pathways dose-dependently. A decrease in spliceosome ribonucleoproteins, an increase in proteins related to acute phase inflammation and lipid metabolism as well as moderate changes in amino acid metabolism were observed with NM200/NM203. Moreover, SAS interfered with phosphorylated proteins, potentially altering stress response signaling. Co-exposure with MG132 suggested that ribonucleoprotein loss was not due to proteasomal degradation, but rather to alternative degradation pathways or reduced expression. This study corroborates that multi-omics supports the detection of early cellular responses induced by nanomaterials and can improve risk assessments of food-grade materials with a nanosized fraction. Disclaimer/:This research received funding from the European Union through a grant of the EFSA (agreement:GP/EFSA/SCER/2020/04). This communication reflects only the author's view and EFSA is not responsible for any use that may be made of the information it contains.

P240
Hepatotoxicity of food ingredient synthetic amorphous silica (SAS) using NAMs: a case study from the NAMS4NANO project

ABSTRACT #117

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The application of New Approach Methodologies (NAMs) represents a significant advancement in the assessment of nanomaterial toxicity. This approach, supported by the European Food Safety Authority (EFSA) via the NAMs4NANO project, follows the recommendations of the EFSA Guidance on nanomaterial risk assessment. The project investigates the implementation of NAMs within Integrated Approaches to Testing and Assessment (IATA) to improve risk assessment and better understand remaining challenges and uncertainties. Among the nanomaterials studied, synthetic amorphous silica (SAS), authorized as a food additive (E 551), was selected for in-depth analysis in a dedicated workpackage. Two precipitated (NM200 and NM14113), one pyrogenic (NM203) and one silica gel (NM14113) materials were supplied from the Joint Research Center repository. These four food-grade SAS, along with the control nanomaterial Sicastar (plain, 20 nm) were tested on HepaRG liver cells cultured in 2D and 3D models. Cells were exposed for 24 hours to a concentration range up to 100 µg/mL with dispersions prepared according to the Nanogenotox protocol. Flow cytometry analysis (granulometry, SSC) and electron microscopy confirmed SAS nanoparticle uptake in HepaRG cells. Assessments of cell viability (ATP), apoptosis (caspase 3), genotoxicity (γH2AX), oxidative stress (DCFDA), and inflammation (interleukin release) indicated no acute toxic effects. Histological analyses were performed on the 3D model, showing that SAS exposure did not disrupt the maintenance of their hepatocyte-specific phenotypic characteristics. These findings highlight the importance of the NAM integration into food nanomaterial toxicity

assessment and contribute to the development of integrated approaches for improved risk evaluation in food safety. Disclaimer : This research is performed under the NAMs4NANO action, which received funding from the EU through a grant of the EFSA (GP/EFSA/MESE/2022/01). This communication reflects only the author's view and EFSA is not responsible for any use of the information it contains.

P241
In vitro study on inhibitory evaluation of hydrogen peroxide using infusions made from black and roselle tea
ABSTRACT #123

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Objectives: Infusions made from some favorite type of teas namely black tea (Camellia sinensis) and rosella tea (Hibiscus sabdariffa L.) content valuable compounds such as galic acid, quercetin and the other polyphenols [1]. The presence of these important secondary metabolites released from above mentioned tea to infusions can have anti-aging, antioxidant, anti-diabetic anti-hyperlipidemia effects as well as preventing cell cycle progress in some cancers [2]. Hydrogen peroxide is believed to create oxygen-derived free radicals which is key role in most of cardiovascular, renal and liver diseases [3]. This study was carried out to evaluate the potential of two known infusions made form black and roselle tea which are consequently abbreviated as BTI and RTI; in contrast to Vit-C for scavenging the hydrogen peroxide in vitro. Methods: Hydrogen peroxide concentrations in test tubes were spectrophotometrically measured at 390 nm using potassium iodide reagent. Briefly the reactions were included of Trichloroacetic acid (0.1 %, 0.5 ml), K – phosphate buffer (0.5 ml, 100 mM), different concentrations of hydrogen peroxide ranged from 0.06 to 1mM, along with abovementioned tea infusions (0.5ml, 5/100 W/V). The percentage inhibition was calculated using the absorbance difference between control and infusions. H2O2 scavenging activities of BTI and RTI were evaluated by calculation of 50% inhibitory concentration (IC50) for each infusion and finally compared with Vit-c value. Results: The IC50 values calculated for BTI and RTI were found to be 1382.84 ± 34.48and 1587.45 ± 49.37 µg/ml, respectively. While the calculated IC50 value for Vit- C was 16.41± 0.02 µg/ml. Conclusion: Based on this study it seems that BT infusion can have better scavenging activity when compared with RT infusion and in this direction may hinder hydrogen peroxide induced cell death in vitro. Key words: Scavenging activity, Hydrogen peroxide, Black tea, Roselle tea, Infusion

P242
Is the Ames screening test predictive for the regulatory Ames test?
ABSTRACT #126

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Before a chemical or pharmaceutical product can be marketed, genotoxicity testing is mandatory. Therefore, it is essential to screen lead compounds for genotoxic properties during the early development and scale-up phases using in vitro assays. The singular agar plate Ames screening test is suitable for this purpose, requiring 4–10 times less test material than the regulatory Ames test. However, its predictive value for the regulatory outcomes has not been fully validated. In this study, 100 regulatory Ames tests were retrospectively evaluated as if conducted in Ames screening format, and the results were compared to determine the sensitivity and specificity of the screening assay. The balanced dataset used contained results from 50 negative and 50 positive regulatory Ames tests. These data were evaluated by comparing the study outcomes based on 3 replicate plates per concentration (regulatory format) with the singular first plate (screening format). All 50 positive regulatory Ames test were correctly predicted as positive in the screening format and only 2 of the negative regulatory Ames tests were incorrectly predicted as positive, demonstrating a sensitivity of 100% and specificity of 96%. In conclusion, the singular agar plate Ames screening assay is highly predictive for the regulatory Ames test with no false negatives in this dataset. These results

strongly support its use as a reliable early screening tool.

P243
Detection of DNA cross-links in TK6 cells using a modified version of the in vitro alkaline comet assay
ABSTRACT #128

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Under the EU REACH regulation, which is regulated by the ECHA, the safety testing and data requirements are dependent on the tonnage level of the chemical substance produced and imported. For genetic toxicology assessment, the initial study required from a one-tonnage threshold onwards is the bacterial Ames mutagenicity test. A unique positive response in tester strain TA102, may indicate DNA cross-linking properties of the substance, prompting ECHA to request a modified in vivo Comet assay able to detect DNA-cross linkers which may not be detected in a standard Comet assay. However, the exact protocol needed, robustness and reproducibility of this in vivo method remains uncertain. As an alternative approach, an in vitro modified comet assay has been developed to detect cross-linking agents more reliably. Human lymphoblastoid TK6 cells were treated with the cross-linking agent cisplatin (12.5 - 1600 µM) for 3 hours. Cells were collected in cold mincing buffer and comet slides were prepared. Following lysis, the slides were treated with various concentrations of methyl methanesulfonate (MMS) to induce DNA damage prior to subsequent electrophoresis under standard alkaline conditions and scoring of slides. The DNA migration was quantified as % tail intensity. MMS treatment of the slides induced a dose-related and pronounced increase in DNA migration in TK6 cells, while cisplatin treatment alone did not affect DNA migration. In MMS-treated slides, cisplatin produced a robust, dose-related decrease in DNA migration compared to control cultures, consistent with DNA cross-linking activity. These findings demonstrate that the in vitro modified Comet assay in TK6 cells can reliably detect DNA cross-linking agents like cisplatin. The magnitude of the observed response reflects the robustness of this assay. which is a robust alternative to the modified in vivo Comet assay requested by ECHA.

P244
Investigation of early metabolomic indicators of drug-induced liver injury(DILI) using extracellular untargeted metabolomics
ABSTRACT #150

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The liver is a major metabolic organ that plays a central role in the biotransformation of endogenous and exogenous compounds. Among these, pharmaceuticals are of particular concern due to their high reactivity and increasing diversity, which can lead to drug-induced liver injury (DILI). Current clinical assessments of DILI, such as tissue biopsy and blood component analysis, are limited by delayed detection and long diagnostic times. In vitro long-term hepatotoxicity assays also require extended durations, typically around two weeks. This study aims to establish a cost-effective and repeatable in vitro diagnostic approach that enables earlier detection of DILI based on biomolecular changes in cultured hepatic cell line(HepaRG). The underlying hypothesis is that changes in the levels of biomolecules derived from the cell line, reflecting mechanistic differences, correlate with subsequent hepatotoxicity. HepaRG cells were exposed to three DILI and three non-DILI drugs at high and low concentrations. After five days, sample preparation of cells and cultured media was performed and analyzed using LC-QTOF-MS/MS in non-targeted mode. Statistical analysis with MS-DIAL and MetaboAnalyst 6.0 revealed biomolecular differences between control and exposed groups. Pathway analysis further suggested potential biomarkers associated with hepatotoxic mechanisms. These findings are expected to provide a basis for indentifying early-stage biomolecular indicators of DILI and non-DILI hepatotoxicity

P245
Hazard identification with alternative methodologies: a benzophenone-based photoinitiator disturbed bile acid homeostasis in human liver cells

ABSTRACT #155

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Background and Objectives Benzophenones (BPs) are widely used as photoinitiators for paper and board-based food packaging materials, but some BPs have limited toxicological information, such as 4,4'-bis(diethylamino)-benzophenone (DEAB), 4-phenylbenzophenone (4-PBP), and 4-methylbenzophenone (4-MBP). The objective of our present study was to identify the toxicological endpoints of these BPs and understand the mode of action using altermative methodologies. Material and Methods: In silico tools, including ToxSTAR and DILI Predictor, were applied to predict hepatoxicity. Three human liver cell models were used: HepG2, HepaRG and induced pluripotent stem cell-derived human hepatocytes. Transcriptomic and bioinformatic analysis followed with the real time RT-PCR assay were applied to examine the effects on gene expression and biological pathways. Change in protein expression was confirmed with immunostaining. Intracellular bile acids concentrations were determined with the the triple quadrupole mass spectrometry. Results: In our present study, we predicted that BP, DEAB, 4-PBP, and 4-MBP might exhibit hepatotoxicity using in silico methods. However, we found that only DEAB was cytotoxic in human hepatoma cells HepG2. Transcriptomic and biological pathway analyses showed that DEAB treatment significantly disturbed bile acid synthesis pathway. Our transcriptomic analysis revealed that DEAB decreased the expression of bile acid synthesis-related genes cytochrome P450 7A1 (CYP7A1) and Acyl-CoA oxidase 2. We further validated the expression of these genes through the real-time PCR assay in HepG2, HepaRG cells as well as induced pluripotent stem cell-derived human hepatocytes. We further confirmed the increased CYP7A1 proteins using immunocytochemistry in all three human liver cell models. Finally, we demonstrated that DEAB decreased bile acid concentrations, including chenodeoxycholic acid, glycochenodeoxycholic acid, and cholic acid in HepG2 cells. Discussion and Conlclusion: Our study highlights the successful use of alternative methodologies to prioritize chemicals of concern and emphasizes the need to evaluate the toxicity of chemicals detected in human specimens in the future.

P246
New approach methodologies (NAMs) as first tier in an integrated approach to testing and assessment for non-genotoxic carcinogens
ABSTRACT #157

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Assessment of the carcinogenic potential of chemicals is a key component of human health risk assessment. Traditionally, cancer hazard identification for industrial chemicals relied on long-term rodent carcinogenicity studies. Under current regulations, such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), risk assessment primarily depends on genotoxicity tests and sub-chronic repeated dose toxicity studies. While these methods have proven effective in protecting human health from genotoxic carcinogens, there is no regulatory requirement to specifically identify chemicals that may cause cancer through non-genotoxic mechanisms (NGTXCs). To address this gap, an alternative testing strategy, like an Integrated Approach to Testing and Assessment (IATA), is needed that are mechanism-based and incorporate New Approach Methodologies (NAMs). Such testing strategy aims to minimize animal use and improve the detection of NGTXCs by combining information from different non-animal methods. In this case study, we selected a diverse set of 29 chemicals covering a range of non-genotoxic modes of action relevant to carcinogenesis. We explored the applicability of various NAMs to determine their suitability for

inclusion in the first tier of an IATA for NGTXCs. The selected NAMs included in silico tools, such as QSAR models and ADME predictions, and high-throughput in vitro assays, e.g., ToxCast and CALUX. The study demonstrates the complementary strengths of these NAMs, but also highlights the need for additional assays to ensure broad mechanistic coverage of carcinogenic pathways. As such, the case study nicely contributes to a more defined composition of a first tier for an IATA for NGTXC.

P247
Estimation of intra-specific ranges of genetic susceptibility to toxicity using genetically diverse populations of human lymphoblastoid cell lines. ABSTRACT #183

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Aim of the study is to provide a greater understanding of the genetic sources of variation that underlie individual susceptibility to toxicity. Consequently, we have carried out high throughput screening of thirteen chemicals (Cadmium chloride, Sodium (meta)arsenite, Acrylamide, 4-Methyl imidazole, Dexamethasone, Cyclosporin A, Tamoxifen, Genistein, Rotenone, Tetraethylthiuram disulfide, Valproic acid, 5-fluorouracil and DMSO) in 261 lymphoblastoid male and female cell lines from 18 populations to discover genetic variability and sex-dependent changes in toxic responses in humans. Clear response differences to all toxicants were found and ANOVA analysis of the cell line dose-response toxicity data from the populations revealed significant intra-population sex differences and population specific responses to the toxicants studied. Further aim is to evaluate the degree of certainty in causal links between molecular key events and chemical risks to human health. Thus, the role of single nucleotide polymorphisms in the toxic responses will be studied using a genome wide association analysis and expression quantitative trait locus mapping is carried out to reveal phenotypic variation in the cell lines. It is commonly considered that pathways predictive of human toxicity are restricted to closely related species (i.e. mammals). By measuring variability in gene expression among non-sentient organisms (NSOs), we will demonstrate that toxicity responses are shared in distantly related organisms. Omics experiments are planned to confirm that the genetic regions controlling variation in molecular key events observed in humans also control variation in the fruit fly. With these findings we hope to promote the use of NSOs to predict human toxicity in regulatory risk assessment. The study is conducted within the H2020 funded PrecisionTox project (Grant agreement ID: 965406).

P248
High-throughput RNA sequencing reveals distinct transcriptomic responses of human jurkat T cells to cyanotoxins and their binary mixture ABSTRACT #185

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Cylindrospermopsin (CYN) and microcystin-LR (MC-LR) are cyanotoxins increasingly detected in aquatic environments due to climate change and eutrophication, raising concerns about human exposure effects. Although their immunotoxicity has been reported, their impact at the molecular level remains poorly characterised. We investigated the transcriptomic alterations induced by CYN, MC-LR and their mixture in the human Jurkat T-cells. Cytotoxicity was evaluated using the MTS assay to determine appropriate sub-cytotoxic concentrations for transcriptomic analysis via

RNA sequencing. Differentially expressed genes (DEGs) were identified based on adjusted p-values (<0,05) and fold change>1,15 and then performed Gene Ontology (GO) and KEGG pathways analysis using DAVID Bioinformatocs Resources. Results of Functional Annotation Clustering indicate that the mixture induced a distinct transcriptomic profile compared to each toxin administered individually. So, the mixture showed activation of processes related to the protein synthesis machinery, as evidenced by enrichment of GO terms associated with mitochondrial translation and ribosome biogenesis. In contrast, the mixture showed significant repression of sterol and cholesterol biosynthesis pathways. Notably, the mixture exhibited more FDR-significant enrichments processes among the upregulated genes than the downregulated genes suggesting a predominantly activation-oriented transcriptomic response. Compared with CYN alone, the mixture upregulates genes involved in adaptive immune response processes, while no FDR-significant enriched pathways were identified among the downregulated genes. Compared with MC-LR, the mixture upregulated genes associated with mitochondrial translation, while downregulated genes were enriched in biosynthesis and amino acid transport. Furthermore, CYN and the mixture caused more widespread changes in gene expression than MC-LR, affecting a variety of cellular functions. These findings help to understand the molecular mechanisms of immunotoxicity induced by cyanotoxins. Keywords: Cylindrospermosin; Microcystin-LR; Jurkat cells; Transcriptomic Acknowledgement: This work was supported by the MICIU/AEI/10.13039/501100011033 and FEDER project PID2023-147444OB-I00. R. F. B. and A. C.-R. acknowledge the Spanish MICIU/MICINN for the predoctoral grants PREP-2023-001191 and PRE2020-094412 respectively.

P249
Evaluation of the neurotoxicity of biocide isothiazolinone
ABSTRACT #186

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Isothiazolinones (ITs) are biocides with various biological properties and are hence used across different industries. They are commonly found in consumer products, disinfectants, and household cleaners. Although effective, ITs have been reported to exhibit neurotoxic effects in mouse- and rat-derived cells. However, the neurotoxicity of ITs in humans remains underexplored. In this study, we compared the neurotoxicity of four commonly used ITs—1,2-benzisothiazol-3(2H)-one (BIT), 2-methyl-4-isothiazolin-3-one (MIT), 2-n-octyl-4-isothiazolin -3-one (OIT), and 4,5-dichloro-2-octyl-4-isothiazolin-3-one (DCOIT)—in human-derived neuronal cells. The viability of cells exposed to these ITs decreased the most with DCOIT, followed by OIT, BIT, and MIT, respectively. The ITs induced changes in neurite outgrowth and the expression of neurodevelopmental genes at concentrations considered noncytotoxic. RNA-sequencing revealed the regulation of the expression of genes associated with neurodevelopmental processes; ANXA2 and SYTL5 were upregulated following treatment with all four ITs, indicating their potential as biomarkers for IT-induced neurotoxicity. A Kyoto Encyclopedia of Genes and Genomes pathway analysis indicated an association between all the tested ITs and neurodegenerative diseases. BIT caused the most pronounced changes in neurodevelopmental gene expression, with the highest potential for neurotoxicity. These findings provide insights into the potential neurotoxicity of ITs and highlight the need for revised safety assessments and regulatory strategies to address the risks associated with human exposure. This work was supported by Korea Environment Industry & Technology Institute (KEITI) through Technology Development Project for Safety Management of Household Chemical Products Program, funded by Korea Ministry of Environment (MOE) (2022002980005, 1485019201) and the Korea Institute of Toxicology (KIT) Research Program (no. 2710008763, KK-2501-01).

P250
Human relevance assessment of adverse outcome pathways and associated NAMs
ABSTRACT #197

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The human relevance of toxicological pathways, i.e. adverse outcome pathways (AOPs), is often assumed in regulatory risk assessment in view of a precautionary principle. However, there are examples where effects observed in animal studies are not deemed relevant for humans and are, therefore, disregarded in a chemical substance's risk assessment. Yet, how the relevance of an AOP to humans is to be evaluated remains wide open to interpretation. Similarly, assessing the relevance to human health risk assessment of new approach methodologies (NAMs) that can be associated with toxicological pathways is not always clear. Therefore, we have developed a pragmatic workflow to assess the human relevance of AOPs and associated NAMs. This workflow, and accompanying guidance and templates, support a structured and transparent assessment of human relevance including a proposal for the integration of the collected evidence following a weight-of-evidence approach. We suggest a framework that follows specific biological considerations to categorize the evidence for human relevance. In parallel, it offers a framework that follows specific empirical considerations to evaluate NAMs for their power to provide 'human relevant' data. Additionally, principles for categorization and a quantitative weight-of-evidence is offered to integrate the evidence. The workflow was subjected to several case studies and subsequently revised to demonstrate its applicability and increase its function. We further foresee incorporation of (semi-)automated tools to facilitate a systematic and transparent information search and reporting, visualization of the evaluation results, and recurring refinement of the workflow following the application of the workflow. This work is a next step toward a harmonized approach for human relevance assessment of AOPs and associated NAMs. Guidance to assess the relevance of NAMs, specifically in the context of one or more elements of an AOP, will help to further advance these methods toward regulatory acceptance.

P252
Transcriptomic–based analysis to discriminate non-genotoxic carcinogens from genotoxic carcinogens and non-carcinogens in a human HepaRG spheroid model: mechanistic insights and biomarker identification
ABSTRACT #205

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Carcinogenic compounds are generally categorized into two classes, genotoxic (GTxC) and non-genotoxic carcinogens (NGTxC). While GTxCs can be detected based on a battery of in vitro and in vivo genotoxicity assays, detection of NGTxCs remains based on 2-year in vivo rodent studies that are time consuming, expensive, pose ethical concerns, and may not be directly extrapolated to humans due to species-specific responses. Thus, it is urgent to develop in vitro New Approach Methodologies (NAMs) to detect NGTxC. In this context and as a part of the PARC project, we applied a NAMs-based in vitro transcriptomic approach using 3D human-hepatic HepaRG spheroids to identify the changes at the molecular level in relation to different NGTxC Modes of Action (MoA). 3D HepaRG spheroids were exposed for 72h repetitive treatment with three different groups of chemicals (A) five GTXs: B) five NGTxC and C) five non-genotoxic non carcinogens at concentrations corresponding to the IC10 and IC10/2. Clustering analysis and the Gene Ontology (GO) enrichment analysis were applied to classify the different groups of chemicals based on their gene expression profiles, and to identify the key molecular events associated with the NGTxCs. So far, the analysis of the results obtained show clusters where some of the tested NGTxC chemicals were clearly show similar gene profile of which are enriched with overexpression of nuclear receptor genes, and genes associated to lipid metabolism. In addition, down expression to genes related to caspase activity were enriched with the gene expression profiles of tested NGTxC. However, further analyses of this preliminary data are needed to confirm the

preliminary data found based on the global clustering analysis.

P253
Characterization of the performance and sensitivity of deiodinase 1 inhibition bioassay using different in vitro models
ABSTRACT #206

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Deiodinase 1 place a major role in thyroid hormone (TH) metabolism and homeostasis. Reduced DIO1 activity alters local TH availability, which can influence signaling pathways, known to drive important developmental processes, but also cancer progression. Suppressed DIO1 activity leads to decrease of intracellular T3 levels, which often favours a less differentiated, more proliferative phenotype in cancer cells. Currently, an assay for the assessment of the ability of chemicals to interfere with DIO1 activity using human liver microsomes is being internationally validated. Nevertheless, their use raised concern among the OECD expert group regarding possible variability among the sources of these microsomes, their availability for testing and comparability of results. Another study used transiently transfected cells, where the content of viral particles limits the wide use of these cells. Also, a high amount of expressed active DIO1 is crucial to enable the use of a non-radioactive detection. Therefore, the objective of this study is to address this gap and establish a novel stable in vitro model overexpressing DIO1 for a non-radioactive detection assay, respecting 3R principles, suitable for routine testing of TH disruptive potential of chemicals, their mixtures as well as environmental samples. We have conducted a detailed analysis to characterise the suitability of different in vitro models for assessing DIO1 inhibition. Namely, we have compared the functionality and sensitivity of models based on endogenous expression and stably transfected models, including the detectability of deiodination and its inhibition using the Sandal-Kolthoff reaction. Stable transfection based on the HEK293T cell line, free from viral particles, enabled setting up such assay, allowing easy transferability to other laboratories with standard equipment. Also the cells with the endogenous expression enabled the detection of DIO1 activity and its inhibition by model chemicals. Nevertheless, in that case much greater amounts of proteins were needed to enable sensitive detection.

P254
Establishment and optimization of thawing procedures for murine non-parenchymal liver cells as progress of primary hepatic co-culture systems
ABSTRACT #209

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At PRIMACYT Cell Culture Technology GmbH, we performed our first comprehensive study on the thawing and characterization of primary murine non-parenchymal liver cells (NPCs), including Kupffer cells, sinusoidal endothelial cells, and stellate cells. These cell types play key roles alongside hepatocytes, which are already a standardized model for toxicological studies. Our aim was the establishment reproducible culture conditions for murine NPCs, enabling the development of primary cell-based liver constructs that closely mimic the in vivo hepatic microenvironment and serve as the foundation for future 2D and 3D co-culture systems. Isolated and cryopreserved primary murine NPCs were compared to different thawing methods containing PRIMACYT's NPC Maintenance Medium, an Albumin-based buffer and RPMI medium. Cell viability, recovery, and cell type-specific functionality were assessed, along with antibody fluorescence microscopy identification of each cell population. The results show that Kupffer cells tended to show the best attachment and viability when thawed using the NPC Maintenance Medium. They displayed strong particle uptake activity and positive CD68 antibody staining. Stellate cells had significantly higher viability and recovery when thawed with the Albumin-based buffer. They formed retinoid lipid droplets and also proliferated over time. Sinusoidal endothelial cells demonstrated improved recovery and viability when thawed using the albumin or RPMI buffer compared to the NPC Maintenance Medium. In conclusion each NPC subtype requires its own optimized thawing

approach for successful recovery and plateability. This study provides to our knowledge one of the first demonstrations that primary murine NPCs can be effectively cryopreserved and thawed, which is a crucial step toward establishing advanced co-culture and 3D liver models using primary cells. Future experiments will be done on establishing the murine models co-culturing the NPC with primary hepatocytes enhancing primary liver in vitro models.

P255
Metabolic activities in rainbow trout (oncorhynchus mykiss) S9 fractions liver and extrahepatic organs as an alternative in vitro ecotoxicity assessment approach
ABSTRACT #213

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Whole body biotransformation rate constants can be calculated by using in vitro to in vivo extrapolation (IVIVE) models. These models use in vitro intrinsic clearance CLin vitro, int rates derived with OECD Test Guideline (TG) 319A/B to estimate liver clearance rates in Rainbow trout, which can be extrapolated to a whole-body biotransformation rate constant. Primacyt supports this approach of alternative method to animal experiments. Rainbow trout were maintained under controlled housing conditions according to OECD TG 319A/B. Specimens of eight sexually immature animals (per lot) were obtained and pooled, including liver, gill, intestine, brain, heart and spleen to generate S9 fractions, in total 2 lots. These fractions were prepared to determine the Cytochrome P450 Phase I and Phase II enzyme activities by Liquid chromatography-mass spectrometry analysis. Phase I activities were determined by using 7 compounds focusing on time dependence and distribution among the organs. Glucuronidation and sulfation of 7-OH-Coumarin as Phase II reactions were analyzed as well. Liver and intestine displayed the highest Cytochrome P450 activities of all organs tested. The Phase I formation of four metabolites were linear up to 60 min in intestine and 120 min in liver. Extrahepatic Phase I activities of four enzymes were found in all organs with different specification to the tested compounds. Phase II activities were detected in liver, intestine, gill and heart, but not in brain and spleen. In summary, liver is the major organ for detoxification of compounds in Rainbow trout. Extrahepatic organs, mainly intestine, exhibit certain Phase I Cytochrome P450 activities with different specification to the reference compounds. Phase II enzyme activities were besides liver also detectable in intestine and gill. Our results suggest that extrahepatic organs, mainly intestine, should also be taken into account when bioaccumulation and in vitro clearance rates are determined for IVIVE modeling in Rainbow trout.

P256
Impact of microplastics in ambient air on A549 alveolar cells and RPMI 2650 nasal cells
ABSTRACT #215

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Global plastics production is estimated at 430 million in 2024, and forecasts suggest that this figure is only set to rise [1]. Indeed, the presence of microplastics (MPs) in the environment due to mechanical, chemical, and biological degradation of plastic devices to sizes between 5mm and 1µm is proven. As indicated by the findings of multiple global studies [2], MPs have been detected in the atmosphere, and while we can breathe them in, we currently lack the necessary data to predict their impact on human health. Studies have shown the presence of plastic particles inside our lungs [3], and the evaluation of the toxic effects of these compounds must now be carried out. The detection of MPs in air was accomplished by means of staining air samples with IDye Poly Pink, and their identification was performed by Py-GC-MS. Then, nasal (RPMI2650) and alveolar (A549) epithelial cells were exposed in vitro for 6 and 24 hours to reference MPs detected in air at various concentrations (0.1, 1, 10, and 100 µg/cm²). Several parameters related to cell viability and toxicity (extracellular LDH quantification, Neutral Red Update Assay, and

AlamarBlue), cellular metabolism (lactate and ATP quantification), and inflammation (TNFα, IL-1β, and IL-6) were assessed after exposure. No cytotoxicity was observed with both cell models. However, an alteration of the cells metabolism was noticed, even at low doses (lactate increased, modification of mitochondrial function, …). Indeed, they have a production of IL-6 for A549 and a production of IL-1β for all cells, demonstrating an inflammatory reaction of these cells in contact with microplastics. These preliminary studies highlight the presence in the air of micrometric plastic fragments of various types (polyethylene, polypropylene, polystyrene, etc.). Furthermore, the effects observed on respiratory cells demonstrate the influence of these microplastics, particularly on cellular metabolism and the inflammatory response.

P257
Perimyelintox: developing a peripheral demyelination assay
ABSTRACT #233

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Adult peripheral neurotoxicity remains poorly addressed in current in vitro test batteries. Within the European Partnership for the Assessment of Risks from Chemicals (PARC), project PeriMyelinTox aims to develop a human-relevant assay for detecting chemical-induced peripheral myelin toxicity. A system was established in which human iPSC-derived Schwann cells and motor neurons are co-cultured in 3D spheroids for 4-6 weeks. Myelin is then quantified by immunofluorescence staining of neuronal and myelin-specific markers, βIII-tubulin or neurofilament and myelin basic protein, respectively, followed by semi-automated software analysis. Overall, myelin formation is largely consistent across 3D spheroids, enabling reproducible and higher-throughput analysis. Several factors of interest underwent parallel investigation for implementation in the final assay. One series of experiments was aimed toward fostering myelin output via supplementation of promyelinating compounds in order to maximize assay sensitivity. Substances examined include vitamin B12, cyclic adenosine monophosphate, insulin-like growth factor 1, coenzyme Q10, neuregulin-1, ciliary neurotrophic factor, and laminin. Congruently, myelin expression was observed on a weekly basis over a 10-week period with the intent to quantify myelin over time and identify crucial windows for observing different outcomes, such as myelination, demyelination, and recovery. Once optimal myelination conditions were defined, possible positive and negative control substances regarding peripheral demyelination were screened based on existing literature. Next, the assay will be applied to chemical-induced demyelination and validated with a blinded, standardized compound set from the European Joint Research Center, forming a master data set across international participants of the PARC project. This platform provides a novel, human-relevant approach for peripheral neurotoxicity testing in next-generation risk assessment and offers new ways of observing and manipulating peripheral myelin in a laboratory setting.

P258
Potency-response (PR) plots: a new tool for the visualization of complex data sets
ABSTRACT #246

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New Approach Methods (NAMs) can improve genotoxicity assessment by providing insight into the genotoxic mode of action (MOA) of novel substances and materials. NAMS, such as ToxTracker, can be used to identify various direct and indirect genotoxic effects by looking at the activation of different reporter genes. Differential activation of these reporter genes inform about DNA reactivity as well as indirect

genotoxicity related to induction of oxidative stress, protein unfolding and cytotoxicity. To move beyond genotoxicity hazard identification and advance to risk assessment, incorporating potency assessment is crucial. Genotoxic points of departure can be estimated via benchmark dose (BMD) modeling, which produces lower (BMDL) and upper (BMDU) confidence bounds for ranking potency, while also displaying a measure of variation. While BMDs are typically calculated for a single endpoint, ToxTracker uses six multiplexed GFP reporter genes to predict genotoxic MOA. This work aimed to create a tool visualizing both BMDs and response strength across multiplexed datasets. BMD analysis (using Proast v71.1) was applied to ToxTracker data from 21 DNA synthesis inhibitors (nucleoside analogs) across six ToxTracker cell lines. Compounds, spanning decades of development, were grouped into three generations. “Potency-Response” (PR) plots displayed potency (BMDLs) and GFP response magnitude. Unsupervised hierarchical clustering on response grouped compounds by MOA, revealing three clusters: genotoxic substances (n=11), oxidative stress inducers (n=3), and negatives (n=7). Seven of ten Gen 1 compounds were in the genotoxic cluster, while two of three Gen 3 compounds induced predominantly oxidative stress, illustrating the safety evolution of this chemical class. PR plots simplify interpretation of multiplexed data, combining potency and quantitative response information. This approach supports risk assessment based on in vitro data, enhancing the utility of NAMs in regulatory decision-making.

P259
Determination of the genotoxic and chemopreventive properties of beta-sitosterol on human peripheral lymphocytes using the comet assay
ABSTRACT #260

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Beta-sitosterol is a plant-derived phytosterol compound and a member of the sterol family. Plant sources of beta-sitosterol include fruits, vegetables, vegetable oils, nuts, grains, and legumes. Research conducted on beta-sitosterol has demonstrated that the substance exhibits antimicrobial, anti-inflammatory, antibacterial, antioxidant, anticancer, and antidiabetic properties. In this context, it is crucial to investigate the genotoxic and antigenotoxic potential of beta-sitosterol. In the study we conducted, the genotoxic and antigenotoxic potential of the beta-sitosterol compound was investigated in human peripheral lymphocytes using the Alkaline Single-Cell Gel Electrophoresis (COMET) assay. Method In the genotoxicity analysis section of the study, human peripheral lymphocytes were exposed to five concentrations of beta-sitosterol (4.88, 9.75, 19.5, 39, and 78 µg/mL) for 1 hour. To determine the antigenotoxic potential, five concentrations of beta-sitosterol and H2O2 (100 µM) were applied simultaneously to the lymphocyte cells for a 1-hour exposure period. The obtained data were evaluated using the Comet Assay IV software package (Perceptive Instruments Ltd., UK), based on tail intensity and tail moment parameters. As a result of the statistical evaluation, it was observed that the beta-sitosterol compound had no genotoxic effect on human lymphocyte cells at any concentration. Additionally, it revealed a strong antigenotoxic potential against H2O2-induced DNA damage. In this context, it is of great importance to conduct new studies using different testing methods on various model organisms to more clearly reveal the genoprotective effects of the beta-sitosterol compound. Keywords: Phytosterols, Beta-sitosterol, Genotoxicity, Antigenotoxicity, COMET assay Acknowledgement: This study was supported by Gazi University Research Fund under the project number FYL-2025-10465.

P260
Standardizing and automating high throughput organoid culture and downstream applications with the mo:re lab platform
ABSTRACT #261

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Human organoids are three-dimensional structures derived from stem cells that mimic the architecture and function of human organs, enabling the study of physiological and pathological processes in vitro. However, their manual production and screening often result in pipetting errors and variability, limiting their reliability in pre-clinical research. To address this, Mo:re developed the MO:BOT, a laboratory automation platform designed to standardize organoid culture and downstream processing. In this project, the MO:BOT was applied to the high-throughput production and screening of liver spheroids, and kidney and cardiac organoids derived from induced pluripotent stem cells in 96-well plates. The system automated cell seeding, medium exchanges, and image-based morphological evaluations. Selected organoids were then subjected to automated cell viability testing and to drug toxicity screening, while manually prepared organoids served as controls. The MO:BOT generated homogeneous 3D aggregates, accurately performed medium changes, and maintained organoid area, roundness, and solidity. Organoids produced with the MO:BOT exhibited higher viability compared to those made manually. In endpoint analyses, liver, kidney and cardiac organoids were exposed to compounds at varying concentrations, demonstrating their suitability for in vitro toxicity testing. In conclusion, the MO:BOT enables fully automated generation and analysis of 3D organoids, improving reproducibility, scalability, and traceability in pre-clinical research. This technology represents a significant advancement toward standardizing organoid-based assays for high-throughput applications.

P261
Comparative evaluation of in silico and in vitro tools for the prediction of metabolites in livestock studies ABSTRACT #270

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Human safety testing for novel pesticide active substances traditionally relies on a series of in vivo studies, including livestock assessments to evaluate residue levels in edible commodities. In alignment with the 3R principle - reduce, refine, and replace animal experiments - new approach methodologies (NAMs) present a promising opportunity for enhancing early-stage predictions of livestock metabolism and kinetics. This study investigated the efficacy of various in silico and in vitro techniques for predicting metabolites in gallus gallus, a key model species for livestock metabolism. A series of experiments was conducted using three test substances at concentrations of 5 or 10 µM. These were incubated with common in vitro liver systems, specifically sub-cellular fractions (microsomes and S9), primary hepatocytes in suspension, and hepatocyte spheroids. The samples were analyzed using high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) to elucidate the metabolic degradation patterns. The resulting metabolite data was systematically compared with predictions generated by in silico tools, including SyGMa and BioTransformer, as well as findings from conventional in vivo studies. A scoring of the metabolites predicted based on the number of hits in independent tools was discussed and the overall predictivity of the different techniques evaluated. Although the NAMs investigated showed inherent limitations in predicting the metabolic pathways observed in vivo, our findings suggest that an integrated approach combining in silico and in vitro data can effectively complement in vivo study results. This integrative strategy holds significant potential for advancing our understanding of metabolism in livestock and improving the safety assessment process for novel pesticide active substances.

P262
Comparing natural toxins and pesticides: A broad targeted metabolomics analysis in HepaRG cells ABSTRACT #274

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Background: Human exposure to synthetic and natural contaminants through diet remains a public health concern. Pesticides and natural toxins, such as cyanotoxins or mycotoxins, can exceed tolerable exposure limits and exert toxic effects, often targeting the liver. Metabolomics offer comprehensive analyses of metabolic changes following toxicant exposure to identify perturbed pathways. This study compares metabolic profiles of HepaRG cells – a human liver cell model with metabolic capacities closely resembling primary human hepatocytes – exposed to a pesticide and two natural toxins to identify compound-specific and shared responses, providing mechanistic insights and demonstrating the value of in vitro metabolomics in hazard assessment. Methods: HepaRG cells were treated with azoxystrobin (AZX), enniatin B1 (EB1) and microcystin-LR (MC-LR) for 24 h at concentrations between IC1 and IC75. Broad targeted metabolomics by LC-MS/MS was performed as described by Ramirez-Hincapie et al. (2023). Cells were extracted in-plate and analysed by reversed-phase and hydrophilic interaction chromatography with MS/MS detection. Pathway analyses were performed at IC5 and IC30. Results and Discussion: In total, over 250 unique analytes were quantified, and 114-160 differentially expressed metabolites identified per test-substance. Principal component analysis indicated two distinct modes of action for AZX, with a clear separation between lower (IC1, IC5) and higher (IC30) concentrations. In contrast, MC-LR and EB1 showed gradual and consistent concentration-dependent effects up to IC30. Among other classes, triacylglycerols were significantly affected: AZX caused a strong and consistent increase in triacylglycerols, MC-LR a pronounced and consistent decrease and EB1 comparatively moderate, less consistent effects across this class. These findings demonstrate the suitability of HepaRG models for metabolomic investigations of hepatotoxicity and demonstrate the approach's sensitivity in capturing compound-specific and shared metabolic perturbations. The observed responses highlight the central role of lipid metabolism in chemical-induced liver toxicity and support the potential application of metabolomics in mechanistic hazard assessment.

P263
Drosophila melanogaster as a model to investigate the effects of environmental contaminants on the gastrointestinal microbiota
ABSTRACT #284

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The widespread generation of micro- and nanoplastics (MNPLs) during industrial and manufacturing processes has raised growing concerns about their potential effects on human health, particularly under conditions of repeated and chronic ingestion. While most studies have focused on the toxicological impacts of MNPLs on respiratory and gastrointestinal health, their influence on the human microbiota remains poorly understood. However, investigating this relationship is challenging due to the vast diversity and complexity of microbial communities in humans. Therefore, alternative animal models have been proposed as intermediate systems to help elucidate MNPL–microbiota interactions. Among these, Drosophila melanogaster (the fruit fly) has emerged as a valuable model organism, widely used in diverse research fields thanks to its genetic tractability, short life cycle, and well-characterized microbiota. Thus, we first aimed at identifying the developmental stage at which D. melanogaster exhibits the highest stability in gut microbiota composition—an essential step given the lack of consensus on the optimal age for microbiota-related investigations in this species. Later, we assessed the impact of MNPL exposure on the gut microbiota composition and diversity of D. melanogaster using high-throughput sequencing approaches. Preliminary results show significant alterations in both alpha and beta diversity between control and exposed groups, indicating that MNPL exposure disrupts the microbial community structure. Such dysbiosis may contribute to downstream effects on immune regulation, metabolism, and systemic inflammation. Overall, these findings highlight the relevance of D. melanogaster as a reliable model to study the effects of environmental contaminants on microbial communities. Moreover, we emphasize the need for further research into

MNPL–microbiota interactions, including the elucidation of mechanistic pathways and the identification of microbiota-derived biomarkers for exposure monitoring and early detection of potential health risks.

P264
Optimization of exogenous metabolic activation system for enhanced detection of nitrosamines using MultiFlow and MicroFlow assays
ABSTRACT #306

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Background: Nitrosamines such as NDMA, NDEA, NNK, NMOR, NPYR, NNN, and NDELA are established mutagens and carcinogens that require metabolic activation to elicit genotoxic effects. Standard in vitro assays and exogenous metabolic activation show limited sensitivity or inconsistent detection of such compounds. Recent advances show that hamster-derived S9 may provide more appropriate activation and Oniell et al 1982 reported on the benefits of the addition of excess of calcium chloride as a cofactor. This study aimed to optimize nitrosamine detection in the context of MultiFlow and MicroFlow assays. Materials and Methods: TK6 human lymphoblastoid cells were exposed to at least 10 concentrations of each nitrosamine under several metabolic activation conditions. A standard continuous low-dose exposure with 0.25% S9 was compared to short-term conditions incorporating 3% rat S9, 3% hamster S9, or a 1.5% rat + 1.5% hamster combination. $CaCl_2$ was introduced according to Oniell. At 4 and 24 hours, cells were evaluated for γ-H2AX, phospho-Histone H3, and p53 using MultiFlow, and for micronucleus frequency using MicroFlow at 24hr. Data were acquired by flow cytometry and benchmark dose (BMD) metrics were calculated using PROAST software. Results: The optimized 3% S9 $CaCl_2$-enriched systems produced stronger and more consistent concentration-response relationships compared with the 0.25% continuous exposure. Species-related activation patterns were observed with the rat system providing the most robust activation for bulky nitrosamines while the hamster system preferentially activated smaller methylating nitrosamines. The combined Rat + Hamster S9 condition captured the full spectrum of nitrosamine reactivity within a single exposure system. Discussion and Conclusions: Across optimized systems, genotoxic biomarkers signal intensities increased markedly, producing BMD rankings consistent with known in vivo carcinogenic potency. The optimized S9 activation system enables robust potency ranking and mode-of-action characterization, supporting integration of MultiFlow and MicroFlow assays into next generation IATA frameworks for assessing metabolically-activated genotoxicants.

P265
Refining gold standard in vitro models for the NAMs era
ABSTRACT #314

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In vitro cell models are central to ADMET research and important for drug development, yet many widely used systems still rely on animal-derived components that limit their reproducibility and 3R compliance. We have explored how established human-relevant in vitro models can be refined to achieve completely animal product-free conditions without compromising their functional performance. Primary human hepatocyte spheroids represented a commonly used model for studying hepatic metabolism, toxicity, and drug clearance. We have recently shown that a chemically defined, animal product-free medium could replace fetal bovine serum (FBS) during spheroid formation while maintaining appropriate morphology and viability. Over extended culture periods, key hepatic functions including cytochrome P450 activity were preserved, and global proteomics indicated a high degree of similarity to conventional cultures, supporting their suitability for ADMET applications. We also investigated the Caco-2 cell line, widely regarded as the gold standard model for intestinal permeability. Several clones were successfully adapted to the animal product-free medium, and although these cultures showed a somewhat longer doubling time, they retained their monolayer integrity and the ability to predict intestinal drug absorption that are essential for a functional permeability

model. Interestingly, global proteomics demonstrated more in vivo like expression levels of several ADMET-related proteins, findings that were also reflected in functional assays. Together, these advances support the feasibility and value of transitioning ADMET-relevant cell models to fully animal product-free culture systems, improving reproducibility and advancing ethical approaches in drug development.

P266
In vitro genotoxicity – investigation of different mechanisms using micronucleus test via flow cytometry
ABSTRACT #322

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The in vitro micronucleus (MN) assay is a OECD-guided standard test tool for evaluating the genotoxicity of compounds in risk assessment. However, it is time-consuming and involves the use of different hazardous compounds during the test protocol. In order to interpret the data obtained from the MN assay appropriately, additional cytotoxicity tests are required. An alternative method is flow cytometry (FCM), which, in addition to detecting micronuclei, enables the detection of cytotoxic events and cell cycle distribution. This method was established by Bryce et al. (2007) (1) using cell lysis and staining of DNA with SYTOX Green. Prior staining with ethidium monoazide bromide (EMA) allows differentiation between living and apoptotic/necrotic chromatin. The micronuclei of living cells are distinguished from the cell nuclei based on their size and DNA content. The measured DNA content of the cell nuclei can be used to generate cell cycle data, providing additional information on the mechanisms of action of the test compounds. Further mechanistic evidence may be provided by the population of hypodiploid cell nuclei. An increase in this population is indicative of aneugenic chromosomal damage. (1) Verification of the „aneugenic gate“ and examination of cell cycle distribution after cell lysis are performed using various substances with calstogenic and/or aneugenic effects and comparison with the established MN assay according to OECD TG 487 (2). Using various natural substances and the antineoplastic substances etoposide, mitomycin C and nocodazole, it is demonstrated that FCM reliably assesses genotoxicity. In addition, FCM is characterised by high throughput, thus providing improved statistical reliability and allowing the invesitgation of a wide concentration range. In sum, the established test protocol provides an effective way of screening (natural) compounds as well as their mixtures in vitro, offering initial insights into their respective underlying mechanisms in the onset of gentoxicity.

P267
From insights to impact: acceleration of NAM implementation by policymakers
ABSTRACT #337

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Background: The transition towards animal-free safety assessment of chemical substances requires the effective implementation of New Approach Methodologies (NAMs). Despite a growing number of NAMs being developed, their validation and acceptance within legislation and regulatory frameworks remain challenging. Policymakers and advisors, both nationally and internationally, play a crucial role in stimulating this process of which they are often unaware. Moreover, they may often lack sufficient insight into the NAM implementation process to formulate effective policy activities. Recent discussions, e.g. in the context of the implementation of the European Commission Roadmap phasing out animal testing, have highlighted the need to identify where policymakers and advisors can more effectively

contribute to the acceleration of this process. Material and methods: To address this, an infographic was developed to summarize and visualize the NAMs implementation process for policymakers and advisors. The infographic was built in two phases. First, an extensive literature review was performed to identify per stage of NAM implementation the associated policy-relevant drivers and barriers. Second, the resulting infographic was tested with a broad range of stakeholders, mainly policymakers and advisors, whose feedback was used to refine the final version. Results and conclusion: The infographic can be used to raise awareness among actors involved in policy making on how they can take action to accelerate the transition toward animal-free safety assessment.

P268
The concise summary of the country reports of the Asian federation of societies for alternatives to animal experiments (AFSAAE)
ABSTRACT #347

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AFSAAE, established in 2024, links societies in Japan, India, China, the Republic of Korea, and Sri Lanka to advance the 3Rs (Replacement, Reduction, Refinement) and non-animal methodologies (NAMs). Because member countries differ in economic and technological capacity, region-wide information has been fragmented; AFSAAE therefore compiles concise country reports covering regulatory readiness, research capacity, education, and industry engagement. Overall, NAM adoption is increasing: spanning in vitro assays, computational modeling, and advanced human-relevant platforms such as organoids and organ-on-a-chip, yet implementation remains uneven and validation/acceptance pathways vary across jurisdictions. In China, the Society of Toxicological Alternatives and Translational Toxicology (TATT) has organized expert working groups and drafted national R&D reporting; regulatory momentum is reflected by the National Medical Products Administration (NMPA) encouraging alternatives for cosmetic safety assessment (2025), alongside standards development and international coordination (including OECD-related activities). In Korea, the Ministry of Food and Drug Safety (MFDS) advances international standardization, contributing to OECD test guidelines and achieving ISO recognition for reconstructed human tissue–based irritation methods; KSAAE supports national dissemination and cross-sector networking. In India, the New Drugs and Clinical Trials Rules (2019) mandate validated alternatives where applicable; SAAE-India is pursuing a national validation center with government support to expand research and training and reduce unnecessary animal use. In Sri Lanka, SAAT highlights needs for stronger legislation, updated ethical review, and alignment with international guidance, while noting progress integrating laboratory animal welfare into national law. In Japan, JSAAE promotes 3Rs through training and scientific exchange, and JaCVAM supports method evaluation, standardization, and international acceptance; remaining priorities include sustained funding and broader regulatory uptake. Common barriers include limited funding, insufficient validation infrastructure, and lack of harmonized regulatory acceptance and awareness; AFSAAE emphasizes regional cooperation to accelerate NAM development, validation, and implementation in Asia.

P269
In vitro safety assessment of locally produced biosimilars and herbal products in Pakistan using 3D liver and

kidney microstructures
ABSTRACT #356

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Background: Pakistan has a growing biosimilar industry. Herbal and traditional products are also widely used. However, safety data for many of these products are limited. Standard 2D cell cultures often fail to mimic real human organs. Advanced 3D liver and kidney microstructures provide a more realistic way to detect early toxicity. Objective: We aimed to evaluate the safety of selected locally produced biosimilars and commonly used herbal formulations. We used 3D liver and kidney models to detect liver and kidney toxicity. Methods: Three biosimilar monoclonal antibodies and three herbal formulations were tested at multiple concentrations for 72 hours. We measured cell health using ATP levels, LDH release, and imaging of mitochondria. Results were compared with standard 2D liver and kidney cultures. Results: The 3D models were more sensitive than 2D cells. Biosimilars A and B showed minimal toxicity. Biosimilar C caused mild stress in liver cells. Among the herbal formulations, Formulation A caused mild liver toxicity. Formulation B showed early kidney stress. Formulation C appeared safe in both liver and kidney models. Conclusion: 3D liver and kidney microstructures are effective for testing the safety of locally produced biosimilars and herbal products in Pakistan. Using these models can help identify potential toxic effects early. This approach can support safer medicines and better regulatory decisions. Keywords: Biosimilars, herbal products, liver toxicity, kidney toxicity, 3D microstructures, Pakistan, in vitro safety

P270
From Static 2D Cultures to Dynamic 3D Intestinal Tissues: A New Strategy for Di-GIT Prediction
ABSTRACT #357

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Drug-induced gastrointestinal toxicity (DI-GIT) is a common, dose-limiting adverse effect and remains difficult to predict in preclinical testing, leading to significant late-stage drug attrition in pharmaceutical development. The small intestine, responsible for absorbing ~90% of orally administered drugs and performing first-pass metabolism, is often the first site of therapeutic action but also of injury (1). Predictive DI-GIT remains limited because human and animal in terms of absorption, distribution, metabolism, and excretion differ substantially (2), and conventional 2D models, such as Caco-2 monolayers, fail to accurately mimic human intestinal physiology, exhibiting altered differentiation, deficient metabolic enzymes, including CYP3A4, CYP2C9, and CYP2C19 (3), and supraphysiological TEER values (300-2400 Ω·cm²) compared to native tissue (12-120 Ω·cm²) (3). To address these constraints, Caco-2 cells are co-cultured with mucus-secreting HT-29 cells; however, static platforms inherently lack physiological mechanical cues. To overcome these limitations, there is a transition to human-relevant 3D models (e.g., EpiIntestinal™) and dynamic platforms (e.g., MIVO). We aim to systematically compare Caco-2 monolayers, Caco-2/HT-29 co-cultures, and 3D organotypic tissues under both static and dynamic flow conditions. Yet, the predictive performance of intestinal models has not been systematically compared across different levels of physiological complexity using integrated mechanistic endpoints. Key endpoints include cellular viability, barrier integrity (TEER), cytokine profiling, immunohistochemistry, and glycosylation. Evidence demonstrates that 3D organotypic tissues accurately reproduce native architecture and metabolic competence, achieving >80% accuracy in predicting diarrhea-inducing drugs (3). Furthermore, integrating dynamic luminal and capillary flow in microfluidic platforms accelerates epithelial maturation from 21 days to 7-10 days, enhances ZO-1 tight junction expression, and triggers the formation of physiological 3D villi-like structures (4). Overall, this work supports physiologically relevant 3D human intestinal tissue models as promising preclinical tools for more predictive, human-relevant assessment of pharmaceutical and toxicant safety and efficacy.

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P271
Standardizing sample preparation and delivered dose in complex in vitro models
ABSTRACT #377

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Background and Objectives Complex New Approach Methodologies (NAMs) are increasingly applied in safety assessment across sectors. However, their reliability depends on exposure realism, sample preparation, and dosimetry. Poor control of aggregation, instability, adsorption, or matrix interactions may decouple nominal from effective dose, reducing reproducibility and regulatory interpretability. This work identifies key preparation challenges in particulate and chemical testing and proposes a harmonized workflow to strengthen in vitro hazard assessment. Material and Methods Applied in vitro studies were performed using polymer-based particulate materials and low-solubility or reactive chemicals. Dispersion behavior was assessed under biologically relevant conditions, and solvent choice, pre-conditioning, and handling were systematically evaluated. Exposure control and delivered-dose considerations were aligned with the OECD Guidance on Sample Preparation and Dosimetry for Manufactured Nanomaterials (2025). Organ- and route-relevant NAMs were considered, including air–liquid interface (ALI) systems and in vitro digestion prior to intestinal testing. Chemical characterization (composition, purity, stability, and dispersion/solubilization behavior) was integrated with biological testing to support interpretation of bioavailable and delivered dose. Results Preparation was frequently the main limiting factor. Polymer materials showed distinct dispersibility: stable dispersions were achievable for some (e.g., PLA), whereas others (e.g., PP, PET) formed persistent aggregates that constrained assay suitability. In vitro digestion prior to intestinal testing altered particle behavior and changed biological outcomes versus non-digested materials, including shifts in cytotoxicity and genotoxicity signals. Responses also varied across cellular models, with different cell lines showing different sensitivity patterns. Overall, toxicity could shift across exposure stages and test systems, supporting multi-point evaluation under realistic exposure and dosimetry conditions. Discussion and Conclusion Standardized preparation, dosimetry, and exposure paradigms are essential for robust NAM implementation. Integrating chemical characterization with in vitro methods improves mechanistic interpretation, enhances inter-laboratory reproducibility, and increases confidence in regulatory hazard and safety assessments.

P272
A decision tree framework to restrict animal testing as a last resort: A case example for acute oral toxicity assessment in the European union
ABSTRACT #392

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Background and objectives: Many global chemical regulations require the use of animal testing only as a last resort, meaning in vivo studies should be conducted only when necessary to support safety decisions and when no suitable non-animal methods are available. Despite this mandate, animal testing is still frequently performed for endpoints where validated and regulatory-accepted non-animal methods already exist, underscoring the need for clearer guidance and greater regulatory accountability. Building on the Animal-Free

Safety Assessment (AFSA) Collaboration's analysis (Macmillan et al., 2024), AFSA has launched a project to develop decision trees (DTs) that provide a transparent framework to guide regulatory decision-making and support compliance with last-resort provisions. Methods: These DTs integrate waiving principles, existing regulatory guidance, and relevant non-animal methods to ensure that state-of-the-art science is systematically incorporated into risk assessment processes. Four priority regulatory endpoints were identified: acute oral toxicity, skin and eye irritation, and skin sensitisation, and expert working groups were assembled to develop each DT and outline steps toward regulatory implementation. An overview of the approach, initially formulated during the initiative's kick-off workshop, has recently been published and serves as the basis for the ongoing development of decision trees (Willett et al., 2025).Results: To set a regulatory precedent and refine the framework, a detailed DT for Acute Oral Toxicity has been developed and evaluated for its applicability under the European Union's chemical regulation, EU REACH. The approach for populating DT modules with appropriate testing and non-testing strategies will be presented, accompanied by illustrative case studies demonstrating how the DT ensures that all relevant non-animal-based strategies are considered before resorting to in vivo acute testing. Conclusion and outlook: By strengthening transparency, accountability, and integration of non-animal methods, this initiative aims to accelerate the transition to non-animal safety assessments and support broader regulatory adoption.

P273
Refining the adverse outcome pathway (AOP) for radon-induced lung cancer: integrating genetic variability and environmental co-exposures to improve risk characterization
ABSTRACT #397

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Background and Objectives Radon is the second leading cause of lung cancer worldwide and a major environmental carcinogen. Current risk assessment models and Adverse Outcome Pathways (AOPs) rely on population-averaged relationships and do not adequately incorporate inter-individual biological variability or co-exposures such as tobacco smoke. Evidence suggests that genetic variability in DNA repair pathways and environmental co-exposures modify biological responses to radon-induced DNA damage. This study aimed to refine the radon-induced lung cancer AOP by integrating mechanistic evidence on genetic susceptibility and tobacco smoke co-exposure to improve biological accuracy and risk characterization. Material and Methods Two scoping reviews were conducted using PubMed and Embase to identify mechanistic, molecular, and epidemiologic studies examining genetic variability in DNA repair pathways and radon–smoking co-exposure. Eligible studies included human, animal, and in vitro investigations evaluating radon exposure, DNA damage response, genomic instability, and lung cancer outcomes. Extracted evidence was systematically mapped onto key events within the radon AOP framework to identify susceptibility-modifying mechanisms. Results Radon exposure induces DNA double-strand breaks, the molecular initiating event in lung carcinogenesis. Variability in DNA repair genes, including ATM, BRCA1, and XRCC1, influenced repair efficiency, genomic instability, and cancer risk. Individuals with impaired DNA repair capacity exhibited greater chromosomal damage and increased lung cancer risk at comparable exposure levels. Tobacco smoke acted as a multi-stage effector, increasing oxidative stress, DNA damage, inflammation, and repair inhibition, resulting in synergistic effects on lung cancer risk. Discussion and Conclusion Integrating genetic susceptibility and tobacco smoke into the radon AOP improves mechanistic accuracy and regulatory relevance. The refined AOP supports improved risk characterization and identification of high-risk populations, advancing biologically informed risk assessment and targeted public health interventions.

P274
Miniaturized Ames test systems for nitrosamine mutagenicity screening: A systematic evaluation of miniaturized Ames test formats as sustainable alternatives to conventional genotoxicity testing

ABSTRACT #407

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Background and Objectives Nitrosamines are a class of potent mutagens of growing regulatory concern, given their widespread occurrence in finished pharmaceutical products, excipients, and drug and food packaging materials (Ref1, Ref2, Ref3). To address the need for more efficient and sustainable genotoxicity screening tools, this study aimed to systematically evaluate two miniaturized Ames test formats as alternatives to the conventional Petri dish-based assay for the mutagenicity assessment of nitrosamines. Material and Methods The mutagenic potential of 13 nitrosamines was investigated using the Ames MPF (a liquid fluctuation assay) and the MicroAmes6 (a 6-well agar-based test). Both systems were benchmarked against the conventional Petri dish-based Ames assay reported in the literature, as well as against additional genotoxicity endpoints including micronucleus and chromosomal aberration assays. Normalized Lowest Effective Concentrations (nLEC) were calculated to enable cross-assay sensitivity comparisons. Modified protocols were developed to address the influence of solvent selection and test compound volatility on assay reliability. Results The two miniaturized systems demonstrated a high degree of concordance, with 10 of the 13 compounds producing identical overall calls. Where discrepancies arose, they were largely attributable to cytotoxic effects or differences in solvent usage. Both miniaturized formats matched or exceeded the sensitivity of traditional Petri dish assays for the majority of compounds tested, with several substances yielding equivalent nLEC values across methods. Discussion and Conclusion Differences in cytotoxicity profiles across platforms highlight the need for system-specific interpretation of mutagenicity data, and solvent choice emerged as a critical factor warranting careful consideration in assay design. Overall, both Ames MPF and MicroAmes6 were established as reliable, sensitive, and resource-efficient alternatives to the conventional assay. Their reduced compound requirements and methodological flexibility make them well-suited for nitrosamine mutagenicity screening across diverse matrices, including pharmaceutical formulations, packaging materials, food products, and tobacco smoke.

P275
In vitro wound healing model for evaluation of cutaneous re-epithelization
ABSTRACT #429

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Wound healing is a fundamental process to re-establish tissue integrity and skin barrier function. MatTek has developed its own wound healing model created by introducing epidermal wounds in a full thickness in vitro human skin tissue model (EpiDerm-FT) using a punch biopsy. This 3D tissue model consists of normal human epidermal keratinocytes and normal human dermal fibroblasts cultured to form a multilayered model of the human dermis and epidermis. EpiDerm-FT has fully developed basement membrane, and it resembles in vivo skin in regard to both morphology and barrier function. Historically, this model has been used to evaluate re-epithelization of the wound by fixing the tissues, staining with hematoxylin and eosin, and quantifying migration from the wound origin. Alternatively, wound re-epithelization can be visualized in situ by fixing and immunostaining the tissue with markers of epidermal differentiation as well as a marker of fibroblasts. This staining allows simultaneous visualization of migrating keratinocytes (keratin 14), differentiated suprabasal cells (involucrin), and dermal fibroblasts (vimentin) within the wound. Histological and immunohistochemical analysis showed keratinocyte migration at 2 days following wounding. In both methods of evaluation of re-epithelization, wounded tissues cultured without growth factors (2% human serum) had a reduced healing rate in which keratinocytes did not cover the entire wound within a 6 day timeframe. In contrast, wounded tissues cultured with growth factors demonstrated a dramatic increase in healing rate as keratinocyte migration completely covered the wounded area by day 6. In conclusion, the EpiDerm-FT model serves as a valuable tool for studying cutaneous wound healing. It is well-suited model for evaluating

new therapeutic compounds designed to accelerate the healing process and restoring tissue integrity and skin barrier function.

P276
Male reproductive toxicity induced by aminated polystyrene microplastics ABSTRACT #438

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Plastics are widely used materials due to their superior physicochemical properties. However, their inadequate waste management has led to their accumulation across various ecosystems. Over time, plastics undergo fragmentation and degradation into microplastics (MPs), which have recently been detected in different human biological matrices. Aminated polystyrene (APS) is a functionalized polymer widely used in nanotechnology, medical, and commercial applications because it improves the wettability, adhesion, and reactivity of the material. However, APS has been associated with various toxicological effects, including apoptosis, oxidative stress, lipid metabolism disruption, and reproductive and developmental toxicities. The present study aimed to evaluate the toxicity of APS MPs on the male reproductive system. Mouse Leydig (TM3) and Sertoli (TM4) cell lines were used to assess the cytotoxicity, cell death pathways, genotoxicity, inflammatory responses, oxidative stress induction potential, change in testosterone level, and alterations in related gene expression. The results revealed that exposure to APS MPs induced significant cytotoxicity in both cell lines. Comet assay results demonstrated DNA strand breaks in both cell lines. Cell death pathway analyses indicated that APS exposure promoted apoptotic responses and autophagic cell death in testicular cells, suggesting that multiple cell death pathways contribute to the observed cytotoxicity. Increased ROS levels, together with significantly altered oxidative stress-related biomarkers, including glutathione (GSH), malondialdehyde (MDA), protein carbonyl, and 8-oxoguaninelevels, as well as alterations in IL-1α and IL-6 levels, indicated that oxidative stress and inflammatory response constitute some of the underlying mechanisms. These findings suggest potential reproductive toxicity from APS MPs and highlight the increased toxicological risks associated with exposure to these MPs. While preliminary evidence indicates cellular toxicity effects, further research is needed to understand their full impacts on male reproductive health and endocrine regulation.

P342
PLGA–PFCE nanoparticle labelling of autologous t cells for multimodal quantitative cell tracking: translational workflow towards first-in-human use ABSTRACT #441

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Direct, quantitative measurement of immune-cell trafficking in patients is a persistent barrier to early biomarkers and rational optimisation of immuno-oncology strategies. A clinically compatible approach is ex vivo labelling of autologous cells with multimodal nanoparticles (NPs), enabling subsequent in vivo detection without radioactive tracers. PLGA nanoparticles co-encapsulating a non-radioactive ^19F perfluorocarbon (PFCE) and a fluorescent dye (indocyanine green; ICG) provide a single label readable by quantitative fluorescence imaging and background-free ^19F MRI. This work describes the translational workflow and intended application of PLGA–PFCE/ICG NPs for ex vivo labelling of autologous T cells (investigational product: LUMI-T-01) in the context of an early-phase feasibility study in rectal cancer. The process comprises isolation of autologous T cells from venous blood, controlled ex vivo incubation with NPs to drive intracellular uptake, and re-administration of labelled cells followed by quantitative fluorescence molecular endoscopy (including spectroscopy-based quantification) and regional ^19F MRI to assess localisation and detectability. The multimodal design targets complementary strengths: local high-resolution fluorescence quantification within the bowel wall environment and whole-region ^19F signal for background-free detection. Product handling is aligned to clinical workflow and

incorporates standard cell-product release considerations (purity, viability, and labelling efficiency) to support consistent implementation. This NP-enabled, ex vivo labelling concept provides a practical route towards quantitative immune-cell tracking in patients and establishes a foundation for future NAM-aligned validation of performance and safety endpoints in cell-based products. Take-home message: A single PLGA–PFCE/ICG nanoparticle label enables clinically compatible ex vivo T-cell labelling for quantitative fluorescence endoscopy and ^19F MRI tracking. Acknowledgements: EU Horizon/EIC Transition VIVID. Conflicts of interest to be declared.

P343
Designing an integrated strategy for ocular photoirritation evaluation using in silico and in vitro models
ABSTRACT #446

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Ocular photoirritation is an important safety concern for pharmaceuticals and industrial chemicals. Unlike skin phototoxicity, no validated or regulatory-accepted strategy currently exists for the eye. This study aimed to develop and internally evaluate an integrated testing strategy combining in silico and in vitro methods to predict phototoxic effects on ocular tissues. Three reference compounds including chlorpromazine (CPZ), amiodarone (AMD), and 8-methoxypsoralen (8-MOP) were evaluated using a stepwise approach. In silico prediction was performed using three software tools (MLtox, OECD QSAR Toolbox, and DEREK Nexus), which showed model-dependent phototoxic responses, highlighting the importance of applying multiple predictive tools. In vitro evaluation was conducted using 2D cell lines (mouse BALB/c 3T3 and human VH10 fibroblasts) according to OECD TG 432. To better mimic ocular exposure, selected test substances were further evaluated using a reconstructed human corneal epithelium model EpiOcular™. The applied protocol was adapted from OECD TG 498, originally designed for skin phototoxicity and recently applied for medical device testing. Interleukin IL-1α was assessed as a supplementary inflammatory endpoint. All three compounds exhibited clear phototoxic effects in the 2D cell line assays, confirmed by Photo-Irritation Factor (PIF) and Mean Photo Effect (MPE) values. The human VH10 cell line demonstrated consistent results in line with OECD TG 432 acceptance criteria, suggesting its potential as an alternative to BALB/c 3T3 fibroblasts. Phototoxicity was further confirmed in the EpiOcular™ model, although the initial phototoxic concentration varied depending on the solvent used, highlighting the critical influence of formulation on tissue-based assay outcomes. IL-1α levels showed increased production at phototoxic concentrations for CPZ; however, a consistent concentration-dependent response was not observed for all compounds. This integrated strategy demonstrates the value of combining in silico, 2D, and 3D approaches within a weight-of-evidence framework for ocular photoirritation assessment and supports the development of reliable, animal-free safety testing methods.

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P344
In vitro salivary migration of phthalates from pvc toys commercialized in northern Algeria
ABSTRACT #448

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Background and Objectives: Children under 36 months are highly exposed to phthalates through mouthing of toys. DEHP and DINP are commonly used plasticizers in PVC toys, but data on exposure in North Africa are scarce. In vitro salivary migration tests simulate realistic oral exposure and support risk assessment. This study aimed to evaluate the migration of DEHP and DINP from PVC toys commercially available in the northern region of Algeria, focusing on the potential exposure of young children. Material and Methods: Ninety-seven non-compliant PVC toys (phthalate content >0.1% w/w) were selected from local markets. Samples (10 cm²) were immersed in 50 mL of artificial saliva (pH 6.8) and agitated for two consecutive 30-minute periods at room temperature. Migrated phthalates were extracted with n-hexane and quantified by GC MS. Migration rates were expressed in µg/10 cm²/min. Results: DEHP was detected in 44/84 toys (52.4%), with a mean migration rate of 1.54 ± 0.92 µg/10 cm²/min (range 0.11–4.51). Thirteen toys (29.5%) exceeded the recommended guidance value of 1.67 µg/10 cm²/min. Migration rates correlated strongly with DEHP content (r=0.895, p<0.001). DINP was detected in 6/42 toys (14.3%), mean 1.90 ± 1.34 µg/10 cm²/min (range 0.42–4.25), all below the 6.67 µg/10 cm²/min limit. Migration was most frequent in dolls and bath toys, while minimal in animals and balls. Discussion and Conclusion: A significant fraction of PVC toys sold in northern Algeria may expose children to DEHP above safety guidance values via mouthing. DINP migration was generally lower and within recommended limits. The absence of national phthalate regulations in Algeria highlights the urgent need for monitoring and enforcement to reduce chemical exposure risks in young children. Keywords: DEHP, DINP, PVC toys, in vitro salivary migration, children exposure, North Algeria, regulatory gap.

P345
A cross-study multi-organ cell atlas of Macaca fascicularis informed by human foundation model annotation: A resource for translational target assessment
ABSTRACT #457

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Non-human primates (NHPs), particularly Macaca fascicularis (cynomolgus macaque), represent an essential model for the preclinical assessment of biologics. However, mounting regulatory pressure to reduce NHP use, combined with the lack of a unified, well-annotated single-cell atlas, currently limits both target qualification and the mechanistic interpretation of toxicity in this species. To address this critical gap, we assembled and harmonized the largest single-cell transcriptomic atlas of M. fascicularis to date, integrating 2.6 million cells from 30 studies across 57 anatomical regions. Furthermore, by leveraging Universal Cell Embeddings (UCE) and training a KNN classifier on fixed embeddings across datasets, we successfully transferred annotations from the human Tabula Sapiens V2 atlas to achieve consistent, high-resolution cross-species cell type identification across 27 tissues. Our analysis reveals robust conservation of major cell types while simultaneously pinpointing critical species-specific differences that significantly impact translational predictability across use cases. First, the atlas identifies conserved target expression patterns, such as EGFR and CD44, in specific skin cell populations in both human and cynomolgus monkey. Second, it serves as a vital resource for investigating ocular liabilities by mapping antibody-drug conjugate targets across distinct cell populations; notably, we identified TACSTD2, ERBB2, and NECTIN4 as exhibiting considerable expression in corneal and conjunctival cells in both species. Finally, the data uncovers potential translational gaps, most notably the absence of CD28 expression in cynomolgus CD4+ effector memory T-cells, a finding that mirrors the historical TGN1412

failure. By enabling early, cell type-resolved evaluation of cross-species target biology, this scalable framework supports informed decisions on when cynomolgus monkey studies are likely to be predictive, when they may be refined, and when alternative approaches may be more appropriate - aligning with emerging 3Rs and NAMs (New Approach Methodologies) guidance in biologics development. Keywords: cynomolgus monkey, single-cell atlas, cross-species comparison, ocular toxicity, 3Rs, NAMs

P346
Implementation and Validation of the In Vitro Comet Assay on Human Lymphocytes with Future Perspectives on Hepatocyte Models
ABSTRACT #463

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The assessment of genotoxicity increasingly relies on reliable in vitro models to predict how substances affect human health. Human Peripheral Blood Mononuclear Cells (PBMCs) are a gold-standard platform for these studies as they circulate through the whole organism, representing an estimate of the overall exposure. In this work, we implement the in vitro Comet assay on human lymphocytes to evaluate primary DNA damage, such as single and double-strand breaks. Human blood cells are the most widely used model in this field, accounting for over 88% of Comet assay studies, as they are ethically accessible and provide a clear picture of systemic exposure. The value of using PBMCs is further supported by their high reproducibility, as they can be frozen without losing DNA integrity (3). By utilizing human cells, this approach aligns with the 3Rs principle, reducing animal use while avoiding inter-species variability to provide results more relevant to human safety. In this context, our study aims to adapt and validate established protocols from the literature to implement a standardized Comet assay method within our laboratory. By verifying its specificity and reproducibility through significant results on reference positive and negative molecules, we demonstrate that the assay is reliable for toxicity studies. As a next step, the aim is the implementation of this assay on hepatocyte cell lines HepaRG, cells that allow the detection of "indirect" genotoxics that would otherwise require the addition of a metabolic activator. This evolution will provide a global view of the genotoxic potential of metabolized substances.

P347
Assessing polymer bioavailability and grouping for REACH: PEG as a model polymer
ABSTRACT #474

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Polymers have historically been exempt from REACH registration; however, potential revisions under the European Chemical Strategy for Sustainability (CSS) may introduce new data requirements, including analytical, ecotoxicological, and toxicological testing. A key component of the assessment for human health relevant effects is to evaluate systemic bioavailability, which determines the internal dose. This is critical because without bioavailability there is no potential for bioaccumulation and systemic toxicity. Hence bioavailability is forming the basis for deciding whether a polymer requires toxicity testing. Molecular size and molecular weight (MW) are recognized as primary determinants of systemic bioavailability and therefore central to tiered human health assessment approaches for polymers. Polymers usually represent a distribution of different MW species and the proportion of components with MWs < 1,000 Da is considered to be bioavailable whereas larger molecules are not. To corroborate this assumption, polyethylene glycols (PEGs) have been selected as model polymers for the present study. PEGs are water-soluble and offer well-defined chemical structures and controlled molar mass distributions or defined molecular weights. In the present study, we studied metabolic stability and uptake of different sizes of PEG. All PEGs showed no metabolic turnover in human and rat S9 fractions (liver, intestine and lung). Reconstructed models of the human intestine and lung (EpiIntestinal™ and EpiAlveolar™) were used as barriers to study uptake of PEGs. Non-cytotoxicity of the PEG-concentrations

applied in the uptake-studies (1 and 5 mM) in the tissue models was demonstrated. Barrier-crossing was size-dependent in the intestinal model but negligible in the alveolar model. In summary, the results indicate that size-dependent bioavailability can be experimentally addressed using human-relevant in vitro models, supporting human health risk assessment of polymers.

P348
Smarter Safety Testing: Miniaturized Ames Assays and Mechanistic FISH-Complemented In Vitro Micronucleus Assay as NAMs for Nitrosamine Genotoxicity Evaluation
ABSTRACT #475

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Background & Purpose Detecting genotoxic impurities in pharmaceuticals, packaging materials, and consumer and industrial products is critical for public health protection. Nitrosamines have become a major regulatory concern, driving international efforts to improve testing strategies. Regulatory authorities recommend the Enhanced Ames Test (EAT) to assess the mutagenic potential of nitrosamines in vitro. A valid negative EAT result can justify a higher Acceptable Intake (AI) limit, making reliable, sensitive testing methods essential for risk-based decision-making. Methods This study evaluated two miniaturized Ames assay formats as New Approach Methods (NAMs) for nitrosamine genotoxicity testing: the agar-based MicroAmes6 (6-well plate format) and the liquid-based Ames MPF microplate fluctuation assay. Both formats used tester strains TA100, TA1535, and E. coli uvrA[pKM101], in accordance with OECD Test Guideline 471. Metabolic activation was performed using 30% hamster liver S9, consistent with EAT recommendations. A cytokinesis-block in vitro micronucleus assay complemented with telomere and centromere staining was also conducted to provide mechanistic insight into nitrosamine genotoxicity. Results Strong concordance was observed between the two miniaturized formats, confirming internal consistency. Both showed high correlation with results from conventional Petri dish-based Ames tests reported in the literature, supporting their validity as alternative methods. Notably, the miniaturized assays detected mutagenic nitrosamines at lower concentrations than the Ames test in Petri dishes, indicating superior sensitivity. In vitro micronucleus data complemented the Ames results by elucidating genotoxic mechanisms. Conclusions This study demonstrates that miniaturized Ames assays and the in vitro micronucleus test are promising NAMs for addressing existing data gaps in nitrosamine safety assessment. The generated data will support safety evaluation of nitrosamines and other genotoxic impurities across pharmaceutical and chemical industries, enabling more informed regulatory decisions, improving public health protection, and promoting sustainable laboratory practices.

P349
Impact of bixafen on murine neural stem cell homeostasis
ABSTRACT #477

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Bixafen is a fungicide from the succinate dehydrogenase inhibitor (SDHI) family, widely used in agriculture and recognized for its high environmental persistence. Its mode of action is based on the inhibition of mitochondrial respiratory chain complex II, an enzyme highly conserved across species. Evidence indicates that bixafen lacks species specificity, altering mitochondrial activity in non-target mammals. As neurogenesis is strictly dependent on energy metabolism, this study aims to characterize the neurotoxic risks of bixafen on the developing nervous system using a New Approach Methodology (NAM). We utilized an in vitro model of murine cortical neural stem cells (NSCs). Cellular homeostasis was evaluated through four main axes: mitochondrial functionality, proliferative capacity, maintenance of stem cell identity, and neuronal differentiation potential. Our results demonstrate that bixafen induces early

mitochondrial dysfunction, which leads to a severe disruption of cellular homeostasis. This metabolic stress significantly impairs the proliferative capacity of NSCs and triggers premature differentiation, causing a breach in the maintenance of the undifferentiated stem state. Furthermore, we show that these perturbations directly impact the differentiation process, specifically by altering the development and maturation of the neurons. These findings establish that bixafen interferes with key developmental processes in NSCs by targeting mitochondrial bioenergetics. We are currently expanding this work by investigating the epigenetic remodeling and the inflammatory response triggered by these mitochondrial disruptions. Overall, the observed shift toward premature differentiation and impaired neurogenesis highlights the vulnerability of the developing brain to SDHI fungicides. This work provides essential mechanistic data for the safety assessment of these compounds during the critical perinatal period.

P350
UNRAVELLING NEUROTOXICITY OF PESTICIDES USING A DATA-DRIVEN APPROACH
ABSTRACT #487

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The use of pesticides is common practice in modern agriculture and crop protection. However, their use has clearly shown to impact human health and ecosystems worldwide. Moreover, there is growing evidence showing correlations between pesticide exposure and neurological diseases such as Alzheimer's and Parkinson's disease. Despite this growing evidence for some pesticides, information is lacking regarding their (neuro)toxicity. The aim of this study is to investigate the link between pesticides and neurological disorders using a data-driven approach. From the Comparative Toxicogenomics Database (CTD), curated and inferred chemical-disease relationships were obtained and complemented with a disease enrichment analysis in CTD using transcriptomics data from an in vitro exposure of a cell line or model to pesticides. Out of 27 datasets from NCBI's GEO and EBI's BioStudies the largest study comprising HepG2 exposed to 31 pesticides was selected. CTD showed for 18 pesticides a direct link with neurological disorders, for 10 only revealed inferred evidence and no evidence for the remaining 3 pesticides, anilazine, nitrapyrin and pymetrozine. Transcriptomics analysis using a moderated t-test in limma (absolute FC of 1.5 and p-value <0.05) between exposure and controls resulted in 30, 104, and 535 differentially expressed genes (DEGs) for these 3 pesticides, respectively. Disease enrichment analysis (p-value <0.05) using these DEGs showed significant neurological related diseases for nitrapyrin and pymetrozine. Anilazine did not result in any diseases, probably due to the low number of DEGs. Increasing the number of genes by using only the p-value (282 genes) revealed only cancer as disease category, thus indicating that Anilazine is most likely not neurotoxic. The presented data-driven approach has demonstrated its usefulness for elucidating neurotoxic information about known pesticides. Extending the datasets can further strengthen the approach, before applying it to new, relatively unknown, and/or data poor pesticides.

Regulatory considerations for complex NAMs

P277
Roadmap on phasing out animal testing for chemical safety assessments
ABSTRACT #120

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In its reply to the European Citizens' Initiative "Safe cruelty-free cosmetics – Commit to a Europe without animal testing" (Communication C(2023) 5041), the Commission committed to the development of a roadmap that will outline milestones and specific actions, to be implemented in the short to longer term, to reduce animal testing and that would be pre-requisites for a transition towards an animal-free regulatory system under relevant pieces of chemical legislation (e.g., REACH, Biocidal Product Regulation, Plant Protection Products Regulation and human and veterinary

medicines). The Commission will publish the roadmap in Q1/ 2026 as a communication accompanied by a staff working document describing more details about the proposed actions. At the ESTIV congress, the Commission can report about the first steps undertaken towards the implementation of the Roadmap actions. This roadmap should serve as a structured action plan to accelerate the transition away from animal testing in chemical safety assessments by setting clear objectives and actionable steps. The roadmap intention is to replace all animal testing, including lower species such as invertebrates, while priority should be given to replace the higher, more sentient species such as vertebrates first. The roadmap outlines the key high-level considerations and policy tools required to allow the transition to conducting chemical safety assessments without the use of animals while maintaining or improving protection of human and animal health and of the environment. The roadmap contains recommendations for the transition to non-animal methods, outlines pathways to better coordinate the identification of regulatory needs and to scale up the development, validation, and implementation of non-animal methods or approaches to meet those needs. Lastly, the roadmap proposes an organisational structure to aid with the implementation of the outlined actions and to foster collaboration among EU Member States as well as internationally.

P278
Mechanistically informed in vitro genotoxicity model for complex molecules: NAMs to prevent unnecessary in vivo follow-up tests in regulatory field
ABSTRACT #182

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Background and Objectives REACH Regulation (EC1907/2006) requires conducting in vivo studies to further assess mutagenicity of chemicals when at least one of the standard in vitro tests resulted positive. Synthetic organic dyes frequently test positive in Ames test (OECD TG471), though the underlying mechanisms remain unclear. In a database of 356 dyes, 156 present this outcome; however, follow-up in vivo mutagenicity tests demonstrated that ≈92% were not genotoxic and/or not carcinogenic. This discrepancy emphasises the need for mechanistically informative, human-relevant in vitro approaches to elucidate genotoxicity pathways and optimise decisions regarding in vivo follow-up testing while advancing current regulatory strategies towards non-animal methods. Material and Methods A mechanistic in vitro mammalian reporter-gene assay (ToxTracker assay) was evaluated to provide qualitative data and Mechanism of Action (MoA) information on potentially genotoxic substances. A pilot study was designed to verify the validity of ToxTracker assay in mapping mechanistic responses of complex molecules such as dyes. Mapping molecules based on their genotoxicity mechanisms enables grouping according to biological similarity, providing the basis for Read-Across and significantly reducing in vivo tests. Results ToxTracker well predicted in vivo responses to dye exposure and highlighted the Ames test's high conservativeness compared to mammalian cell tests. ToxTracker provides 78% of concordance to in vivo Comet assay, correctly classifying dyes with no false negatives (100% sensitivity). Compounds classified as genotoxic by conventional in vitro tests were identified as causing oxidative stress, explaining the positive results observed in standard in vitro tests and the absence of in vivo genotoxicity. Discussion and Conclusion ToxTracker is a robust, high-throughput mechanistic screening tool providing MoA information and improving prediction of in vivo genotoxicity. Integrating ToxTracker as an intermediate test between the Ames test and in vivo studies strengthens weight-of-evidence evaluations and aligns with REACH's objective of reducing animal use.

P279
Distinguishing adult and developmental neurotoxicity using new approach methodologies: NAM4NT
ABSTRACT #245

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The implementation of New Approach Methodologies (NAMs) into regulatory neurotoxicity testing remains a major challenge for chemical safety assessment under EU frameworks such as REACH, CLP, and the Biocidal Products Regulation. Within the European Partnership for the Assessment of Risks from Chemicals (PARC), we initiated the project NAM4NT that aims to evaluate the regulatory readiness of NAMs to distinguish between adult (ANT) and developmental neurotoxicity (DNT). This distinction is crucial, as both endpoints are currently treated separately in regulation, yet their biological boundaries remain uncertain. Building on high-readiness assays from the OECD-endorsed DNT in vitro battery (IVB) and methods developed in earlier PARC projects, NAM4NT combines advanced human cell–based systems and zebrafish behavioral assays to elucidate shared and distinct mechanistic pathways of ANT and DNT. The project is structured into three phases: (1) systematic literature and data mining to map adverse outcome pathways (AOPs) and mechanistic domains; (2) data integration from available database sources and hypothesis refinement; and (3) targeted case studies using complementary human and zebrafish NAMs. These include human neurosphere and BrainSphere assays, iPSC-derived neuronal cultures, synaptogenesis assays, and zebrafish-based systems. By integrating mechanistic, phenotypic, and functional endpoints across developmental stages, the project aims to determine whether existing NAMs can provide sufficient specificity and negative predictive value to support regulatory differentiation of ANT and DNT. Close collaboration with ECHA and other stakeholders ensures regulatory relevance and alignment with the “Key Areas of Regulatory Challenge” identified by ECHA in 2025. The outcomes will strengthen confidence in NAM-based neurotoxicity testing and facilitate their implementation into regulatory frameworks, ultimately promoting the reduction of animal testing and improving human-relevant chemical safety assessment.

P280
A whole-mixture prioritisation approach of plant protection products for non-dietary risk assessment using NAMs
ABSTRACT #275

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Plant protection products (PPPs) contain one or more active substances (AS) and various co-formulants. While AS undergo extensive hazard and risk evaluation before approval, PPPs are assessed mainly for hazard assessment. Thereby, regulators may overlook mixture effects arising from potentially toxic co-formulants in non-dietary risk assessment. This study proposes an approach to assess PPPs as a whole mixture by identifying potential mixtures effects between AS and co-formulants using New Approach Methodologies (NAMs). Firstly, specific target organ and absorption, distribution, metabolism, excretion (ADME) in silico tools were applied to prioritise 285 and 215 PPPs, respectively. Secondly, six PPPs were selected for comparative cytotoxicity testing in HepaRG cells. A PPP containing prothioconazole, tebuconazole, and N,N-dimethyldecanamide (NDA) revealed more than additive effects. NDA’s inhibitory effect on CYP2C19 predicted in silico was confirmed in vitro with an IC50 of 0.808 mg/L. Thirdly, In vitro metabolomics was applied to the prioritised PPP, its AS mixture, and its AS mixture with NDA to elucidate hepatotoxicity mechanisms and derive an in vitro point of departure (PoD). A total of 318 unique intracellular analytes in HepaRG cells were identified, annotated and allocated in 13 ontology classes using LC-MS/MS. Principal component analysis (PCA) of the metabolome enabled the derivation of a PoD for all test conditions. Specifically, a PoD of 4 µM was calculated for PPP whereas a PoD of 28 µM and a PoD of 11 µM were calculated for the mixture of AS and the mixture of AS with

NDA, respectively. The PPP's in vitro PoD will undergo in vitro-in vivo extrapolation (IVIVE) to derive an Acceptable Operator Effect Level (AOEL) for the whole PPP. The PPP's AOEL will subsequently be compared to exposure estimates to assess operator risk. This strategy enables the whole mixture assessment of a PPP and the transition from in vivo to NAM-based approaches.

P281
Establishing an adverse outcome pathway for developmental neurotoxicity: inhibition of voltage-gated sodium channels leading to impaired myelination and cognitive dysfunction
ABSTRACT #302

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The rising prevalence of neurodevelopmental disorders and potential chemical contributions demand more effective developmental neurotoxicity (DNT) assessments. New Approach Methodologies (NAMs), combined with the Adverse Outcome Pathway (AOP) concept promoted by the Organization for Economic Co-operation and Development (OECD), integrate non-animal data and link molecular events to adverse outcomes, providing a mechanistic and human-relevant alternative to traditional animal studies. This project aims to establish AOP 489, describing how binding of a compound to voltage-gated sodium channels (VGSCs) during critical windows of oligodendrocyte (OL) development disrupts OL differentiation and myelination, leading to downstream cognitive deficits. This pathway extends the putative EFSA stressor-dependent AOP network for the pesticide deltamethrin, emphasizing OL biology and vulnerable developmental windows. To support this AOP with scientific evidence, semi-systematic literature reviews and weight-of-evidence analyses were conducted to assess empirical support and biological plausibility of the Key Events (KEs) and KE Relationships (KERs). Moreover, public single-cell RNA-seq datasets were analysed to identify VGSC expression in human glial cells for confirming the human relevance of the AOP by identifying OL progenitors as a vulnerable cell type in humans. The collected evidence consistently linked VGSC disruption to impaired OL differentiation and hypomyelination supporting the empirical and biological plausibility and establishing causal relationships between the KEs. Single-cell RNA-seq data further confirmed the human relevance and defined the susceptible phase during development for this AOP. Overall, this study demonstrates the value of integrating in vitro phenotyping with omics data, structured evidence evaluation, and mechanistic reasoning to enhance chemical hazard identification while reducing reliance on animal testing. These insights provide a foundation for future development of interconnected AOP networks, advancing predictive, efficient, and ethical chemical safety assessment.

P282
Standardization of hiPSC-derived intestinal epithelial cell models
ABSTRACT #366

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Food safety evaluation still heavily relies on animal studies, despite ongoing concerns regarding their human relevance, highlighting the need for robust, standardized in vitro models with improved predictive capacity for in vivo outcomes. Although in vitro human cell models have been developed as alternatives to animal testing, many of these models are oversimplified and consist only of a single cell type to represent an entire organ. Human-induced pluripotent stem cells (hiPSCs)-derived intestinal epithelial cell (IEC) models represent more advanced in vitro systems, as they comprise multiple cell types and more closely resemble native tissue, thereby potentially offering improved predictive capacity for food safety assessment. The aim of the present study was to identify and evaluate critical factors that influence the robustness and reproducibility of a hiPSC-derived IEC differentiation protocol, including the influence of donor variation. To this end, three key

aspects were identified: (i) stemness of iPSCs, (ii) differentiation capacity towards the intestinal lineage, and (iii) functionality characterization of the resulting IEC model. These parameters were evaluated using iPSCs derived from five different donors (three female, two male). Stemness was assessed by analysing pluripotency markers and trilineage differentiation potential. Differentiation capacity was evaluated based on barrier integrity and the expression of lineage-specific differentiation markers. To determine intralaboratory variability, these readouts parameters were assessed in minimally two independent studies. The results demonstrated that the differentiation protocol is robust, as all five iPSCs successfully differentiated into IECs forming a tight epithelial barrier comprising all major intestinal cell types, with limited variation between the iPSC donors. Moreover, minimal variation was observed between the independent studies, highlighting good reproducibility. Functionality characterization of the IEC layers is currently being analysed. Taken together, these findings indicate that the hiPSC-derived IEC model is robust and reproducible and holds significant promise for future food safety risk assessment strategies.

P283
Global harmonization and modernization of safety pharmacology to support the adoption of emerging technologies
ABSTRACT #419

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Background & Objectives Released over 20 years ago, the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) S7A guideline has provided a foundational framework for safety pharmacology assessment for more than two decades. However, advances in therapeutic modalities, new approach methodologies (NAMs) and integrated decision-making strategies have highlighted limitations in the guideline's scope, flexibility, and consistency of regional interpretation. As NAMs adoption expands across drug discovery and development, regulatory frameworks must adapt to ensure emerging evidence is integrated in a scientifically robust and operationally harmonized manner. Materials & Methods To address these needs, an international multi-stakeholder workshop was held in February 2026 to align regulatory and industry perspectives in support of a formal ICH S7A revision. The workshop aimed to establish a shared vision and build consensus among regulatory authorities and industry representatives on the potential revision of ICH S7A, with the objective of informing and supporting a formal decision to reopen the guideline. Results A central focus was the incorporation of weight of evidence (WoE) and integrated approaches to testing and assessment (IATA) to support regulatory decision-making, with attention to data confidence and context of use. The integration of NAMs was considered, emphasizing scientifically justified, fit-for-purpose validation and qualification pathways aligned with evolving regulatory expectations. Regulatory and industry perspectives were collected through multiple complementary approaches. A pre-workshop survey captured input on current safety pharmacology practices, key challenges, and expectations relevant to guideline modernization, NAM integration, and the need for greater regulatory clarity and consistency across regions. Discussion & Conclusion Key outcomes included a clear roadmap for bringing the topic of revision to ICH in November 2026. This workshop provided greater clarity on regulatory expectations and pathways for integration of NAMs into safety pharmacology decision-making, supporting more consistent global adoption as technology continues to mature.

P351
FROM OMICS TO POD: TOXICOGENOMIC PROFILING OF NEONICOTINOID AND AZOLE PESTICIDES
ABSTRACT #484

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Modern toxicology is moving away from animal testing - the current gold standard for risk assessment - toward new approach methodologies (NAMs). While in vitro assays are increasingly being adopted, the validation of toxicogenomic tools still lags behind, despite their recognized utility in elucidating chemicals' modes of action (MoA) and deriving points of departure (PoDs). A key obstacle is the lack of validated, standardized approaches for the analysis and interpretation of omics data in regulatory risk assessment. This case study focuses on four pesticides - two neonicotinoids (clothianidin, thiacloprid) and two azoles (imazalil, tebuconazole) - for which 28-day repeat-dose studies have previously been conducted [1, 2]. Kidney and liver tissues retained from these studies were used for RNA extraction and sequencing via RNA-Seq. To evaluate the influence of sequencing platform choice on the results, gene expression from the same RNA isolates will also be assessed using a targeted approach (TempO-Seq). The analysis followed key steps: differential gene expression analysis (in compliance with the R-ODAF framework), dose-response modeling using BMDExpress to derive gene-level BMDs, and the application of rat liver and kidney TXG-MAPr tools to derive PoDs for deregulated gene co-expression modules [3]. Transcriptomic PoDs from in vivo rat studies were compared with the corresponding apical PoDs. Across all four compounds, the largest gene-level changes occurred in liver-derived samples and generally followed a dose-response relationship (except for tebuconazole). Transcriptomic liver PoDs, defined as the 25th percentile gene benchmark dose (BMD) or the lowest pathway BMD, were typically more sensitive than apical liver PoDs, such as the onset of hepatocellular hypertrophy and cytoplasmic degeneration. This case study informs the drafting of the Transcriptomics Interpretation Framework (TIF) in collaboration with EFSA and other regulatory bodies.

Role of 3R centres in the dissemination and implementation of NAMs

P284
Advancing toxicology through microphysiological systems: the 3Rs collaborative's efforts to drive adoption and regulatory confidence
ABSTRACT #151

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Microphysiological systems (MPS) represent a transformative opportunity in toxicology, offering human-relevant, physiologically complex models that can reduce reliance on animal testing while improving prediction of drug safety. The 3Rs Collaborative (3RsC) launched its MPS Initiative in 2021 to accelerate adoption of these technologies through stakeholder engagement, resource development, and regulatory dialogue. Today, the 3RsC-MPS initiative includes over 100 representatives from over 40 institutions, including 33 commercial providers and end-users. Key activities include an online technology hub and corresponding publication hub, educational webinars with the IQ-MPS, and regulatory focused aims. Recent published consensus findings from developers underscore the need for clear roles, responsibilities, and pathways for regulatory acceptance. In 2026, the 3RsC-MPS will launch a project focused on benchmarking and use and attitudes towards MPS in discovery versus development. Furthermore, to address MPS confidence-building in toxicology applications,

the 3RsC-MPS Initiative, in collaboration with FDA-CDER, NICEATM, and the Critical Path Institute, is leading a cross-platform study evaluating nine liver MPS technologies for their ability to predict drug-induced liver injury - a major risk to patient safety, cause of clinical trial failure, and post-market withdrawal. Each platform is independently testing a blinded set of hepatotoxicants and controls, enabling assessment of reproducibility, variability, and predictive performance across systems. This effort aims to generate robust datasets that inform qualification strategies and regulatory guidance, ultimately supporting broader integration of MPS into safety assessment workflows. Overall, the 3RsC-MPS initiative is an important group that is building momentum for using MPS in toxicology. By fostering collaboration, the 3RsC-MPS Initiative is advancing the 3Rs principles of animal research (replacement, reduction, and refinement) while paving the way for regulatory acceptance and improved patient safety.

P285
Centro 3R: advancing 3Rs principles in education, research, and beyond
ABSTRACT #399

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Centro 3R: Advancing 3Rs Principles in Education, Research, and Beyond Chiono Valeria, Grasselli Elena, Anna Maria Bassi, Ahluwalia Arti, Fabene Paolo, Fiore Gianfranco , Mattioli Belmonte Monica, Nicolini Gabriella , Rainer Alberto, Scarfi Sonia, Visai Livia 3R Centre, Inter-University Centre for the Promotion of the 3R Principles in Education and Research, Politecnico di Torino, Torino, Italy. The Italian Centro 3R, founded to promote the 3Rs in university teaching, has formed a collaborative network to share resources and teaching methods. An initial survey assessed researchers' and students' attitudes toward animal experiments and alternatives, revealing that while Italian researchers were aware of the 3Rs, they often viewed animal tests as indispensable and considered replacements as complementary models. Science students, on the other hand, had limited knowledge of animal protection legislation and the 3Rs concept. In response, the Centro 3R's university network introduced courses and credits emphasizing ethics, one-health, preclinical models, invertebrate models, organ-on-a-chip, and in silico models to mainstream replacement through comprehensive education. These courses, initially elective, have tripled in number since 2018 and are in high demand, indicating the success of the pervasive education strategy. Centro 3R, with its mission to promote the 3Rs and responsible research, is actively engaging the next generation of scientists through expanded course offerings and increased awareness. This complements its broader goal of advancing the "One Health" approach and influencing the well-being of people, animals, and the environment, both at the national and European levels. The Center is also striving for institutional recognition and support from the Italian Ministry of Health and the Ministry of University and Research, recognizing its pivotal role in shaping current thinking on critical issues of planetary well-being.

P286
A novel approach for constructing adverse outcome pathways using transcriptomics-based dual-evidence key event identification
ABSTRACT #421

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Constructing adverse outcome pathways (AOPs) from experimental data requires systematic identification of key events (KEs), yet current approaches often rely on manual curation, limiting reproducibility and scalability. Here, we present a novel transcriptomics-driven approach for AOP construction that integrates dose-response modeling with dual-evidence KE identification. As a case study, quaternary ammonium compounds (QACs), including DDAC and BKC, were selected as representative respiratory toxicants. NHBE

cells were exposed to DDAC and BKC, and dose-dependent transcriptomic profiles were generated. Differentially expressed genes (DEGs) were processed through BMDExpress2 to derive benchmark dose (BMD) estimates and Gene Ontology (GO)/KEGG pathway enrichment terms. To map enrichment results to established KEs, two complementary approaches were applied: Method A directly matches GO/KEGG term IDs from enrichment results against KE-associated annotations, while Method B evaluates gene set overlap between enrichment terms and KE-associated gene sets using Fisher's exact test (FDR < 0.05). Only KEs supported by both methods (A ∩ B) were designated as high-confidence KEs. The resulting AOPs revealed that both compounds share a common molecular initiating event (MIE) through interactions with cell membrane components, then diverge into distinct KE trajectories: DDAC predominantly activates DNA damage-mediated pathways, while BKC triggers apoptosis-mediated pathways. Both pathways ultimately converge on adverse outcomes related to respiratory disease characterized by inflammatory cell recruitment. This approach provides a systematic and reproducible framework for constructing AOPs from transcriptomic data, with broad applicability to other chemical exposures. Keywords: Adverse Outcome Pathway, Quaternary Ammonium Compounds, Transcriptomics Funding Source: This work was supported by Korea Environment Industry & Technology Institute (KEITI) through Technology Development Project for Safety Management of Household Chemical Products Program, funded by the Korea Ministry of Environment (MOE) (project number: 202300230430)

Serum-free approaches

P287
Revisiting 3Rs: rethinking replacement and new approach methodologies
ABSTRACT #148

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Background and Objectives: The 3Rs – replacement, reduction, and refinement – have guided ethical animal research for decades. Yet, as new technologies evolve, terms like "cruelty-free," "vegan," "animal-free," and "NAMs" (new approach methodologies) are increasingly used in research, regulation, and marketing, often inconsistently. They may imply the complete elimination of animal use, while animal-derived materials (e.g., sera, antibodies, collagen) are still commonly used. We seek to raise awareness and clarify this misconception and recommend clear definitions in the transition toward truly animal-free science. Methods: Therefore, we recommend standardizing terminology by establishing clear global definitions and promoting transparency to support informed ethical choices. Regulations should be re-evaluated, i.e., through updating OECD Guidelines and related frameworks, to prioritize animal-component-free testing.. To promote animal-free research, funding for xeno-free projects which align with NAMs principles needs to be ensured. Strengthened partnerships between academia, industry, and policymakers, together with a centralized database to document advances and communicate xeno-free replacements, will accelerate ongoing progress. Discussion and Conclusion: Changing the status quo presents significant challenges for industry, academia, and policymakers, requiring both time and the allocation of adequate resources. Establishing a true gold standard for validating animal-free methods and xeno-free cell media can only be achieved through data and insights derived from human-relevant systems. Meaningful progress depends on developing tools that enable us to track advances in replacing animal use in science. We acknowledge these challenges but are confident that growing awareness and a shared vision of our goals provide a strong foundation for future progress.

P288
Advancing in vitro cell culture practices: achieving truly animal-free experiments and scientifically reliable

and reproducible methods
ABSTRACT #149

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Background and Objectives: We have the goal to encourage and disseminate scientific articles with easily implemented information regarding the use of animal-component-free materials in different experimental settings involving cell culture. Our goal is to assist researchers in replacing fetal bovine serum (FBS), basement membrane extracts isolated from mice harboring Engelbreth-Holm-Swarm mouse tumors as well as trypsin and animal-derived antibodies. Material and Methods: To achieve this goal, we organized a research topic at Frontiers in Toxicology, section In Vitro Toxicology, which invited international researchers to submit truly animal-free research from December 2023 until December 2025. So far, the published articles received more than 25,000 views and 6,000 downloads. Results: In this poster we are presenting these works consisting of the following topics: • Animal-component-free culture of human pluripotent stem cells • An explorative study of HepaRG cells in animal-free hydrogels • Proliferation and metabolic activity of fish cells under short-term serum-reduced conditions • Implementing animal-free alternatives in teaching in molecular and cell biology • A review compiling how FBS is being left behind in the pursuit of more reliable science • Towards the standardization of human platelet lysate production for human hematopoietic cell culture • Optimizing Wnt activation in FBS-free organoid expansion media • Advancing humanized 3D tumor modeling using an open access xeno-free medium • A case study of microthrombosis in a human blood-perfused Vessel-on-Chip • Increasing sustainability and reproducibility of in vitro toxicology applications in serum-free cultivated HepG2 cells • Use of serum-free media for peripheral blood mononuclear cell culture and the impact on T and B cell readouts Discussion and Conclusion: With growing attention to the animal welfare implications of laboratory materials, collective efforts are simultaneously directed at ending the use of animals in research for ethical reasons and to strengthen biomedical outcomes.

P289
Self-assembling peptide hydrogels as user-defined synthetic matrices for in vitro modelling
ABSTRACT #165

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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 customisation of the mechanical and biological properties of the matrix to better reflect human biology (Nitsche et al., 2025). 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 customised 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 leukaemia, 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 behaviours 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.

P290
A cost-effective animal-free approach for generating primary human hepatocyte spheroids
ABSTRACT #435

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Formation of primary human hepatocyte (PHH) spheroids requires serum. Foetal calf serum (FCS) is widely used for cell culture, being both nutrient rich and economically accessible. However, the use of FCS remains a source of ethical concern. Human serum (HS) is a readily available alternative; but its high cost prohibits its wider use. Our previous work showed improved PHH spheroid formation in media containing serum and growth factors. To address the concerns on FCS use, we investigated whether growth factors supplementation alone is sufficient for PHH aggregation. Five lots of PHH were plated using media supplemented with either foetal calf serum (FCSm), human serum (HSm) or growth factors (GFm). The cells were cultured for 14 days during which images were taken from day 3 to determine spheroid formation. Our results demonstrate that growth factors alone is not sufficient for PHH spheroid formation. Interestingly, we observed improved spheroid formation of PHH in HSm media compared with PHH from the same lot in FCSm media. Furthermore, PHH from certain lots formed into spheroids in HSm media but not in FCSm media. This demonstrates a potential advantage in using HS over FCS for generating PHH spheroids. To address the high HS cost, we tested whether PHH spheroids can still form at lower HS concentrations, and whether this reduction can be compensated by the addition of growth factors in the media. Our results show that HS can be reduced by at least 50% without affecting PHH spheroid formation. Furthermore, some lots were still able to form spheroids despite a reduction of HS in the media by up to 90% but in the presence of GF. Our work so far offers the potential for a cost-effective animal-free approach for generating PHH spheroids. A comprehensive plan of work is currently underway to further validate our findings.

Toxicokinetics and in vitro – in vivo extrapolation

P251
Inhalation permeability assessment using combined in vitro & in silico approaches
ABSTRACT #107

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Background: Inhalation is a main route of chemical exposure, yet the fate of inhaled chemicals across the pulmonary barrier remains poorly understood due to accessibility issues and limitations of animal models. New Approach Methodologies, particularly in vitro cell culture models are widely used to assess chemical permeability and provide valuable insights into absorption. However, these models often rely on nominal rather than the actual concentrations, leading to inaccurate permeability estimates. Moreover, a lack of harmonization across current approaches limits data comparability and regulatory acceptance. This thesis aims to improve understanding of chemicals fate in permeability systems and to investigate their transport across bronchial (Calu-3) and alveolar (h-AELVi) human lung epithelial models, by integrating in vitro and in silico approaches. Methods: This study compares bronchial and alveolar human lung epithelial cell models, regarding their capacity to form a tight epithelial cell barrier for a 2-week culture at air-liquid interface. Amounts lost due to chemical evaporation, non-specific binding to polymers and abiotic degradation were assessed in a cell-free in vitro permeability system. Rapid Equilibrium Dialysis was employed to determine non-specific binding to medium proteins and cell proteins. Finally, the chemical transport was measured in apical to basolateral for both cell models cultured in ALI. Results: Both Calu-3 and h-AELVi cells formed stable, tight epithelial barrier, after 2 weeks at ALI. Two non-toxic concentrations (1 and10 µM) were used across experiments. The chemical loss in the permeability system varied depending on the chemical. The fraction bound to biological matrices depends on the lipophilicity of the xenobiotics. Despite the observed differences in cell type, the permeability of the two cells used was found to be nearly identical. Conclusion: Neglecting chemical losses underestimate actual permeability. Incorporating biokinetic modeling to account for compound distribution within in vitro systems enhances result accuracy and informs better experimental design.

P291
In vitro study of the biotransformation of cylindrospermopsin using human and RAT liver microsomes
ABSTRACT #139

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Cylindrospermopsin (CYN) is a cyanotoxin produced by certain species of cyanobacteria in freshwater ecosystems, exerting its toxic effects mainly by protein synthesis inhibition and oxidative stress, being toxicity closely linked to its kinetic parameters. Despite the evident relationship between biotransformation and organ-specific toxicity, detailed studies on the metabolism of CYN remain scarce. This work aimed to elucidate the possible biotransformation pathways of CYN using in vitro models. To this end, CYN was incubated (for a maximum of 2 hours) with hepatic microsomes from male and female rats, as well as with pooled human liver microsomes. The metabolites were then identified using Ultra-High Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (UHPLC-MS/MS) and analyzed using Compound Discoverer™ software. Results confirmed the formation of conjugates with glutathione and glucuronic acid. Several other metabolites were also identified, resulting from dehydration, double reduction, hydration, nitro group reduction, oxidative deamination to alcohol or ketone, or conjugation with carnitine, glycine, palmitoyl, or taurine. Despite identifying a wide range of metabolites, their concentrations remained relatively low, with an overall 20% reduction in CYN concentration

observed after 2 hours of incubation. This finding is consistent with previous in silico assays, suggesting that oxidation of CYN by cytochrome P450 enzymes is limited. These results provide valuable insights into the metabolic fate of CYN, highlighting its limited biotransformation and possible persistence in biological systems. They also highlight the importance of using Next-Generation Risk Assessment (NGRA) methods in the initial stages of toxicological research on substances. However, further in vivo toxicological studies are required to fully assess its metabolomic profile, health risks, and environmental impact. Acknowledgements: Project PID2023-147444OB-I00 funded by MICIU/AEI /10.13039/501100011033 and by FEDER, EU. VII PPTI-US 2025 (Use of General Research Services (I.3) and for Research and Knowledge Transfer Management (I.7)). Keywords: In vitro, Biotransformation, UHPLC-MS/MS, ADME.

P292
Two-layer percutaneous absorption integrated in a PBK framework improves plasma concentration prediction for NGRA of cosmetic ingredients
ABSTRACT #145

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In the development of cosmetics, the implementation of animal-free risk assessment, known as Next Generation Risk Assessment (NGRA), is indispensable. Internal exposure prediction using physiologically Based Kinetic (PBK) models is one of the key technologies to achieve NGRA. The construction of PBK models relies on the precise acquisition of kinetic parameters and precise percutaneous absorption parameters are vital for cosmetics exposed topically in human. In this study, we aimed to predict plasma concentrations of topically applied compounds by capturing percutaneous absorption from two-layers; the stratum corneum and the viable layers towards the application to the NGRA for cosmetic compounds. Diffusion and partitioning coefficient parameters for the stratum corneum and viable skin were derived from ex vivo pig-ear experiments using whole-skin and tape-stripped skin preparations. Liver metabolism parameters were sourced from in vitro tests using hepatocytes, while others were estimated based on in silico information. These inputs were implemented in a whole-body PBK model to simulate plasma time–concentration profiles following topical dosing. The prediction precision of plasma concentration was validated against human percutaneous PK clinical studies. For six compounds with human percutaneous PK clinical studies, predictive values of Cmax in all tested compounds and AUC in five compounds were within three-fold difference compared to observed values, suggesting good predictive performance of two-layer model. The two-layer model consistently outperformed the one-layer model, which regards multiple skin layer as a single layer. Furthermore, case study using a cosmetic ingredient (methylparaben) demonstrated that the maximum steady-state plasma concentration predicted by two-layer model was below the threshold values from in vitro hazard endpoints, supporting the safety of systemic toxicity. These findings suggest that incorporating a two-layer mechanistic skin model into PBK simulations improves prediction of systemic exposure from dermal application and provides quantitative internal exposure metrics compatible with NGRA workflows for cosmetics.

P293
Working towards the implementation and acceptance of QIVIVE in NAM-based risk assessment
ABSTRACT #156

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New Approach Methodologies (NAMs) offer promising opportunities to replace, reduce and refine animal testing for risk assessment. When supported by sound mechanistic evidence based on human biology, NAMs increase the human relevance of risk assessment. Central to the use of NAMs is quantitative in vitro to in vivo extrapolation (qIVIVE), enabling the derivation of human in vitro-based points of departure and

subsequently in vitro-based Health-Based Guidance Values. However, the use of qIVIVE in risk assessment introduces extrapolation steps and uncertainties that need to be recognized and, ideally, quantified. We developed a framework that incorporates all extrapolation steps and highlights associated uncertainties related to the selection of in vitro models, the relationship between in vitro effects and in vivo outcomes, and the choice of in vitro concentration metric (e.g., intracellular vs. nominal). Additional uncertainties arise from the selection and parametrization of physiologically-based kinetic (PBK) models, allocation of in vitro dose metrics to the most appropriate PBK compartment, and alignment of in vitro exposure durations with in vivo exposure patterns. The framework was applied to two case studies, demonstrating its utility in identifying and managing uncertainties in NAM-based risk assessment. Currently, qIVIVE is often conducted without comprehensive consideration of these uncertainties and there is no consensus on how to implement qIVIVE for risk assessment purposes. In spring 2026, an international workshop was organized inviting experts from both scientific and regulatory communities, aiming to contribute to a harmonized approach for qIVIVE in the context of regulatory risk assessment and to further refine the framework, identify data and extrapolation gaps, and discuss strategies for uncertainty reduction. The updated framework, incorporating workshop outcomes, will be presented to underscore the importance of early consideration of study and model design, fostering broad scientific and regulatory support, and advancing the regulatory implementation of NAMs in human risk assessment.

P294
In vitro-in vivo response-response relationships for the in vitro based risk assessment of chemical substances
ABSTRACT #179

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In vitro assays and in silico models can be used to facilitate risk assessment of chemical substances, to increase translatability to humans, and minimize the use of animal testing. Within the INTACT project, we focus on quantitative in vitro-in vivo extrapolation (QIVIVE) methods, which are required to derive health-based guidance values (HBGVs) based on in vitro assays. To translate results obtained in vitro with human cells (lines) into HBGVs, extrapolations across two lines are required: (1) extrapolation from in vitro concentrations to corresponding external in vivo doses, and (2) extrapolation from in vitro responses to the in vivo (adverse) effects. The first step is typically supported by physiologically-based kinetic (PBK) models, while the second step can be addressed by deriving a response–response relationship, which describes the relationship between in vitro and in vivo responses. To facilitate the translation of in vitro response to in vivo effects, we derived response-response relationships for substances based on existing in vitro and in vivo rat data. Neuronal activity of rat cortical neurons was measured in vitro using microelectrode array (MEA). These MEA data were compared with in vivo rat data on the adverse effect of seizures, both belonging to the same Adverse Outcome Pathway. An in vitro distribution model and a PBK model were used to translate the in vitro concentrations to equivalent in vivo doses. The resulting response-response relationship indicates that the concentration causing an increase of 15% in neuronal activity in vitro is equivalent to the dose causing mild seizures in the average in vivo animal. This exploratory work demonstrates the feasibility of creating response-response relationships, and the need for extrapolating responses in QIVIVE and chemical risk assessment based on in vitro data.

P295
Evaluating acrylamide developmental neurotoxicty using the adverse outcome pathway strategy
ABSTRACT #181

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There is an increasing concern on adverse health effects of acrylamide (AA) from dietary exposure. Since AA is a known neurotoxicant that pass the placental barrier and has been found in breast milk, it thus raises concern on its developmental neurotoxicity (DNT) potential to humans. Several in vitro and rodent studies have shown that AA generates adverse effects on key neurodevelopmental processes, and there is a need to systematically evaluate these data to reduce knowledge gaps. Herein, we present the first steps in the application of the adverse outcome pathway (AOP) strategy to give a mechanistic description of AA-induced effects on neurodevelopment at different levels of biological organization, to increase awareness of the health risks of AA exposure to women during pregnancy. A total of 2,734 articles on AA and developmental neurotoxicity were retrieved from databases. They were screened using set criteria and appraised for reliability/relevance using the Science in Risk Assessment and Policy (SciRAP) tool. Data associated with AA exposure and DNT endpoints were extracted from the articles that passed appraisal. Some of the identified endpoints and effective concentrations/doses include, impaired neurogenesis, differentiation and proliferation (0.5, 0.1 and 2 mM), decreased neurite outgrowth (0.01 nM – 100 µM), altered CREB activation and BDNF expression (1 and 70 µM), altered neuronal/glia cell ratio (1 µM), axonal fragmentation/swelling, decreased performance/cognitive motivation (5 mg/kg bw/day). To ensure relevance for human exposure, the exposure measurements in experimental studies will be translated to maternal oral doses by in vitro to in vivo extrapolation (IVIVE), using our previously built pregnant women physiologically-based toxicokinetic model (PBTK) for oral AA exposure. With weight of evidence and expert judgement, the endpoints will be manually curated and aligned into a representation of quantitative AOP network, to better unravel the effect of early life exposure to AA and neurodevelopmental outcomes.

P297
Insight: an in silico tool to support chemical fate prediction and NAM-based assay design
ABSTRACT #240

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Selecting appropriate dose levels is a key challenge when designing in vitro toxicological assays in the context of New Approach Methodologies (NAMs). Experimenters often face the dilemma of choosing concentrations representative of environmentally relevant exposure or doses ensuring to explore mechanistic effects. While bioanalytical measurements may help, acquiring exposure

profiles is cumbersome and not always feasible. To address this limitation, we developed INSIGHT model (In Silico Guide for Harmonized In Vitro Testing), a biokinetic model combining a dynamic mass-balance model (adapted from the Virtual Cell Based Assay, VCBA) with a partitioning module derived from the Virtual In Vitro Distribution (VIVD) model. The dynamic flows and partition coefficients were calibrated then validated using an extensive dataset comprising both literature reports and new experimental measurements, covering 42 chemicals and seven widely used cell lines (HepaRG, HepG2, 3T3 Balb/c, PC12, MCF-7, RTgill-W1, and HEK293). The INSIGHT model demonstrated robustness across a range of cell lines when the parameters driving their metabolic activity or functional transport were informed. The present study highlights the central role of logP, which reflects how easily a chemical moves between water and lipids, strongly influences how chemicals behave in an in vitro system. By predicting how much of a chemical ends up in the culture medium, inside cells, or remains at the nominal concentration added to the plate, INSIGHT will help researchers choose the right dose levels and better interpret their results. This improves the link between in vitro findings and expected in vivo exposures (IVIVE), ultimately supporting safer and more informative testing strategies. In doing so, INSIGHT helps bridge experimental work with next-generation, mechanism-driven risk assessment approaches promoted within NAMs. Future developments will focus on improving the model's predictions and applicability domain through the generation of new experimental data, by collaborative efforts between modeling and experimental communities.

P298
Evaluating toxicokinetics in mixture toxicity: identifying potential CYP inhibitors and substrates to establish common kinetic groups (CKG)
ABSTRACT #252

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Recent interest in evaluating mixture toxicity has prompted discussions regarding the adequacy of existing frameworks for assessing mixtures and whether improvements are necessary to better protect public health. Historically, the toxicodynamic approach has been predominant, employing the dose addition concept and focusing on target organ toxicity and mode of action. However, emerging insights suggest a more comprehensive strategy that incorporates toxicokinetic principles into mixture assessments. Braeuning et al. introduced the concept of Common Kinetic Groups (CKGs), which considers toxicokinetic properties such as absorption, distribution, metabolism, and excretion (ADME) and categorizes chemicals based on their potential roles as inhibitors, substrates, or inducers of metabolism. In this study, we present findings from an in silico screening focusing on the most common CYP (Cytochrome P450) enzymes (CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4), which are crucial in xenobiotic metabolism, to explore potential inhibitors and/or substrates within mixtures. We screened over 200 pesticide active substances using three open-access and one commercial predictive tools: SuperCYPs Pred, CYPstrate, ADMETLab3.0, and ADMET Pred, respectively. The predictions were verified in vitro, and binary mixtures were tested to assess concentration/dose addition modeling. By elucidating the relationship between toxicokinetics and mixture toxicity, our research enhances the understanding of chemical interactions and their effects on human health. Incorporating toxicokinetic principles into mixture toxicity assessment not only improves regulatory strategies for risk assessment and management but also aids in making informed decisions regarding chemical safety. Additionally, combining in silico methods with in vitro testing enhances screening efficiency and reduces reliance on traditional in vivo studies, underscoring the importance of computational tools in advancing toxicological sciences and supporting alternatives to animal testing.

P299
Transport and toxicity of indoxyl sulfate and p-cresyl sulfate across physiological barriers

ABSTRACT #281

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Background and Objectives Uremic toxins (UTs), such as indoxyl sulfate (IxS) and para-cresyl sulfate (pCS), are protein-bound metabolites that accumulate in chronic kidney disease (CKD), contributing to neurological and cardiovascular complications. The MOTOX project aims to elucidate UT transport mechanisms, their modulation by CKD medications, and their impact on physiological barriers—particularly the blood-brain barrier (BBB) and renal tubular epithelium—to better understand their role in CKD-related toxicity. Material and Methods We employed two advanced in vitro models: a BBB model derived from stem cells, and a renal barrier model based on transfected RPTEC-TERT1 cells that mimic proximal tubular epithelium. UT transport and toxicity were assessed under both acute and chronic exposure conditions. The contribution of influx and efflux transporters—notably organic anion transporters (OATs) and ATP-binding cassette (ABC) transporters—in mediating UT passage was evaluated using pharmacological inhibitors. Comparative transcriptomic and proteomic analyses are also planned to decipher the effects of UTs and plasma from CKD patients on these biological barriers in vitro. Results Our data did not demonstrate any involvement of efflux pumps in the transport of IxS and pCS across either barrier. However, our preliminary in vitro findings confirm that OATs may mediate the uptake of both toxins in the proximal tubule and also suggest a disruption of the BBB model under uremic conditions. Discussion and Conclusion These findings provide mechanistic insights into UT handling by key physiological barriers and support the hypothesis that OATs contribute to UT transport. Further studies are needed to identify therapeutic molecules capable of modulating UT transport via OATs and to characterize potential alterations induced by the uremic context on transporter expression and function through transcriptomic and proteomic profiling. This work may guide the development of targeted strategies to reduce UT accumulation and mitigate systemic toxicity in CKD patients.

P300
Mass balance is not related to pesticide physicochemical properties and in vitro model suitability for absorption studies
ABSTRACT #282

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Background and Objectives Physiologically relevant human in vitro intestinal and epithelial lung models, in combination with physiologically-based pharmacokinetic (PBPK) modelling, may help to predict absorption of a variety of pesticides. These pesticides varied by physicochemical properties (e.g. structure and LogP). We compared animal-free intestinal barrier models and a lung model for their applicability of selected pesticides (chlorpyrifos, diazinon, dinoseb, endosulfan, and rotenone). Material and Methods: Apical transport of non-toxic concentrations of pesticides to the basolateral side over time across an intact porcine intestine (inTESTineTM) or a monolayer of Caco-2 cells or a primary bronchial epithelial lung model. Results: Only dinoseb could be recovered in all three models above 80-100% of the added amount. Recovery of the other pesticides varied per model, and was 10% for chlorpyrifos, 30% for rotenone, 60% for endosulfan, and 60% for diazenon. Part of the pesticides may bind to

plastic, but pre-coating of cell culture plastics, with bovine serum albumin, did not improve the recovery of for example, rotenone. All in vitro models uses allow the assessment of barrier crossing of pesticides, however, for assessment of their relevancy for human barrier crossing, comparison with human in vivo data would be needed. Discussion and Conclusion: The result contribute to the ongoing discussion to define and refine the chemical applicability domain of in vitro models with regard to epithelial absorption. Pesticides represent a heterogeneous group of xenobiotics whose behavior in biological systems is difficult to predict. Although they are often treated as a single category, and might have similar physicochemical properties such as Log KOW and pKA, these similarities is not reflected by their absorption, in in vitro systems. The VHP4Safety project NWA 1292.19.272 is part of the NWA research program 'Research along Routes by Consortia (ORC)' and is funded by the Netherlands Organization for Scientific Research (NWO).

P301
Next generation risk assessment: an integrated phenotypic profiling and IVIVE approach for plant protection product mixture effects
ABSTRACT #287

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The initiative to eliminate animal testing in human health risk assessment has led to a significant transition towards next-generation risk assessment (NGRA) across various industries and regulatory agencies. NGRA is a human-relevant, exposure-led, hypothesis-driven risk assessment approach that incorporates new approach methodologies (NAMs). Currently, the risk assessment of plant protection products (PPP) predominantly relies on evaluating individual substances based on animal testing. This innovative framework allows for a comprehensive assessment of whole mixtures, addressing the shortcomings of the traditional component-based PPP risk assessment, which could fail to consider interactions that may enhance toxicity. This study aims to explore the feasibility of a NGRA approach that integrates cell painting, a systems toxicology-based NAM, with toxicokinetic models to establish PPP-based threshold values corresponding to in vitro concentration-response data. For this purpose, in vitro phenotypic profiling was conducted using the cell painting plus (CPP) method on a PPP containing two metabolically interacting active substances. The study examined the effect of individual active substances, PPP-equivalent mixture, and the PPP on HepG2 cells through phenotypic profiling. In vitro-in vivo extrapolation (IVIVE) was conducted using the httk package, with relevant parameters and model assumptions for the PPP, to translate benchmark concentrations (BMCs) derived from CPP data to toxicological threshold values. Although the BMCs of individual substances were higher than those of the PPP, the affected cellular organelles were consistent across all scenarios. The predicted PPP-based toxicological threshold value was lower than the acceptable operator exposure level (AOEL). This study indicates that component-based risk assessment may not fully capture complex interactions within PPPs. These findings highlight the significance of integrating NAMs to provide valuable insights into PPP interactions for regulatory purposes and advance NGRA.

P302
Physiologically-based kinetic (PBK) modelling dataset: an open resource to increase accessibility of PBK models for safety assessment
ABSTRACT #291

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Physiologically-based kinetic (PBK) models integrate exposure information, chemical-specific properties, and biological parameters to simulate the time-course of chemicals in plasma or tissues. By quantitatively describing absorption, distribution, metabolism, and excretion processes, PBK models offer unparalleled insight into internal exposure levels, enabling more accurate predictions of potential effects. Such mechanistic approaches support chemical safety assessment across pharmaceuticals, cosmetics, agrochemicals, and industrial chemicals. Despite their

growing importance, PBK model development and application still face major challenges: 1.limited number of available models relative to the vast chemical universe; 2.need for extensive and diverse input data; 3.scarcity of high-quality validation datasets; 4.lack of harmonized evaluation and reporting standards; and 5.limited expertise among users. Recent collaborative initiatives are addressing these barriers, helping to advance science by improving model accessibility, transparency, and reproducibility. Notably, the PBK Modelling Dataset (PMD) [1] - a curated repository containing over 7,500 published models for more than 1,100 chemicals - represents a major step forward, along with our associated KNIME workflow that enables efficient identification and comparison of existing PBK models [2]. This facilitates knowledge transfer and model adaptation for new or existing chemicals where experimental kinetic data are lacking. These advances directly contribute to ensuring safety by enabling scientifically robust, mechanistic risk assessments that can reduce or replace animal testing - placing PBK modelling at the heart of the New Approach Methodologies (NAMs) framework. Ongoing research presented here, showcases the expansion of the PMD with newly published models and demonstrates best practices for model quality evaluation, ensuring consistency and credibility in PBK modelling across a range of applications. This presentation will summarize recent achievements, outline future directions, and highlight how PBK models can serve as key tools in bridging current regulatory science toward a future firmly grounded in NAMs. Acknowledgment: The Humane Research Trust CIO Funding 1203103

P303
In vivo toxicokinetic mechanisms of new psychoactive substances: A narrative review
ABSTRACT #312

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Introduction : New psychoactive substances (NPS) represent an evolving and significant toxicological challenge due to their rapid emergence, structural diversity, and limited toxicological characterization. Although often designed to mimic the effects of controlled drugs, NPS frequently exhibit unpredictable toxicity profiles. Understanding their in vivo toxicokinetics, including absorption, distribution, metabolism, and elimination, is essential for identifying target organs, elucidating mechanisms of toxicity, and supporting risk assessment and regulatory decision-making. Methods : This narrative review covers in vivo toxicokinetic studies published between 2015 and 2025, identified through literature searches in the PubMed and Scopus databases. Experimental animal studies on new psychoactive substances were prioritized, with emphasis on dose–response relationships, tissue distribution, metabolism, elimination, and organ toxicity biomarkers. Results : In vivo studies reveal that many NPS display rapid systemic absorption and high bioavailability, with preferential accumulation in the central nervous system, as well as in detoxifying organs such as the liver and kidneys. Hepatic biotransformation, largely mediated by cytochrome P450 enzymes, plays a pivotal role in modulating toxicity through the generation of active or reactive metabolites. Repeated or high-dose exposure is frequently associated with bioaccumulation, oxidative stress, neuroinflammation, and organ dysfunction. Several studies highlight marked interindividual variability related to dose, exposure duration, and metabolic capacity, contributing to the unpredictability of NPS toxicity. Discussion and Conclusion : In vivo toxicokinetic investigations conducted over the last decade provide critical mechanistic insight into the systemic behavior and toxicity of new psychoactive substances. These data are essential for hazard identification, dose characterization, and toxicological risk assessment, particularly in the context of limited clinical information. Despite advances in alternative methods, in vivo approaches remain indispensable for capturing the complexity of NPS-induced toxicity. Integrating in vivo findings with in vitro and in silico models may further enhance predictive toxicology and support regulatory responses to emerging psychoactive substances.

P304
Cytotoxicity of tea polyphenols and their complex phase ii metabolism

ABSTRACT #328

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Epigallocatechin gallate (EGCG)is the main polyphenol in green tea and is used frequently in dietary supplements. The European Food Safety Authority is concerned about a possible hepatotoxicity and has set the tolerable daily intake for supplements to 800 mg [1]. In slightly alkaline cell culture medium and in the heat of tea preparation, it undergoes oxidative dimerization and forms theasinensin A and D (TSA, TSD) and oolongtheanin digallate (OTDG) [2]. We evaluated the cytotoxicity of these abundant oxidation products individually and stabilized. Cell viability, necrosis, and apoptosis induction were tested by resazurin assay, LDH-assay and microscopy of nuclear apoptotic features. A stronger necrosis and apoptosis induction was determined for TSA and TSD compared to the parent compound. To better understand the toxicokinetics of these substances, we analyzed the formation of phase II metabolites. Starting with porcine microsomes, we could verify mono- and diglucuronidation as well as mono-, di-, and trimethylation by using HPLC-qTOF. Furthermore, by sequentially performing the methylation and glucuronidation, the formation of a heterometabolite was confirmed for EGCG, TSA, TSD and OTDG. The analytical method was then transferred to HPLC-MS/MS to have a lower limit of quantification. The products of the xenobiotic metabolism could also be analyzed in the extracts of the human hepatocarcinoma cell line HepG2. In further experiments, the accumulation of the metabolites in the medium and in cell extracts was monitored over 48 hours. Overall, we can conclude that these substances are taken up into the cells. The experiment points to a higher phase II conversion for EGCG compared to its dimers. Furthermore, the methylated or glucuronidated metabolites reached their peak concentrations at different times, depending on the incubated substance. The results of our study underline the importance of testing the substances individually and improve the understanding of the uptake and metabolism of large polyphenols.

P305
Integration of in vitro toxicity and high-throughput PBK modelling enables prospective prediction of drug-induced liver injury
ABSTRACT #334

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Drug-induced liver injury (DILI) is a major cause of drug attrition and poses a significant threat to patient safety. However, current preclinical prediction methods, including heuristic screening rules, in vitro assays, machine learning models and animal testing, have important limitations. Here, we show that combining in vitro toxicity data with pharmacokinetic information enables high DILI predictivity, and that both exposure and downstream in vivo effects can be generated prospectively using high-throughput modelling. Using cytotoxicity, mitochondrial toxicity and bile salt export pump (BSEP) inhibition data together with pharmacokinetic parameters, we performed a retrospective analysis of 241 drugs with known clinical DILI outcomes. The ratio of maximum in vivo plasma concentration (Cmax) to the lowest in vitro toxicity value strongly correlated with clinical DILI risk and achieved ROC AUC up to 96%. We then tested whether similar performance can be achieved without measured human PK. When Cmax values were predicted in silico using high-throughput physiologically based kinetic modelling, DILI predictivity remained high, with ROC AUC up to 91%. Importantly, we extend beyond risk classification by coupling exposure predictions to dynamic quantitative systems pharmacology modelling of bile acid homeostasis. These simulations translate BSEP inhibition and predicted exposure into time-resolved bile acid perturbations, helping to identify drugs with a mechanistic BSEP driven liability and to explain why some compounds can appear clinically “idiosyncratic” despite measurable in vitro effects. This PBK to QSP link illustrates how the same framework can move from a binary risk label to quantitative expectations of what happens in vivo, when it happens, and which mechanisms drive it. Overall, this integrative, mechanistic approach improves DILI predictivity and interpretability and offers an

animal-free alternative for early drug development.

P306
High-throughput PBK modelling for systemic PK predictions after dermal exposure
ABSTRACT #335

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High-throughput physiologically based kinetic (HT-PBK) modelling offers a mechanistic approach to estimate human systemic pharmacokinetics (PK) from non-animal inputs and is increasingly considered in next generation risk assessment (NGRA). In this work, we examined the applicability of HT-PBK modelling to dermal exposure, a major route of human contact with pharmaceuticals, consumer product ingredients, and industrial chemicals. Using the skin permeation model implemented in the Open Systems Pharmacology Suite, coupled to a whole-body PBK model, we simulated plasma concentration-time profiles for 52 compounds with published human dermal PK data. Model parameterisation relied exclusively on in silico predictions of physicochemical properties (including lipophilicity, solubility, melting point, boiling point, and vapour pressure) generated by quantitative structure-activity relationship (QSAR) tools, without compound-specific in vitro measurements. We systematically compared alternative QSAR sources and their combinations to identify parameterisation strategies that improve predictive performance. Across the compound set, the best-performing strategy reproduced 75% of observed Cmax and AUC values within one order of magnitude. A tendency toward overprediction of systemic exposure was observed, consistent with incomplete reporting of study conditions and the default assumption of fully hydrated stratum corneum in simulations. Prediction errors were larger than those reported for oral HT-PBK applications, reflecting additional variability and complexity in dermal absorption processes and study protocols. Overall, these results indicate that fully in silico HT-PBK modelling can provide screening-level estimates of key systemic exposure metrics following dermal application, supporting exposure-driven assessment approaches when experimental kinetic data are limited.

P307
Complex distribution phenomenon of test chemicals in cell culture experiments: exemplification by tebufenpyrad
ABSTRACT #355

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To predict the toxicity effects of chemicals, it is important to consider that toxicants are not usually freely dissolved in the aquatic environment, such as cell culture media, cells, and body fluids, but are partially bound to biomolecules; only a fraction of the chemical is free/unbound (fu). Consequently, the free and nominal concentrations and how they differ from each other are determined by test system factors (e.g., the protein and lipid content, and the type of surface material). Here, we aimed at obtaining experimental data on the distribution of tebufenpyrad (TEBU) within the culture dishes (medium, plastic, and protein fractions) and LUHMES cells. Such data can be used to provide better IVIVE and/or to reduce uncertainty with respect to a relevant PoD. In protein-free medium (DMT), only about 25% of nominal TEBU was recovered, corresponding to a loss of 75%. When 1% BSA was added (DMBT), recovery increased to about 33%, corresponding to a loss of 67%. Furthermore, we investigated whether the presence of protein would have an impact on TEBU's neurotoxicity. The results showed that protein supplementation shifted TEBU-induced effects on neurites and cell viability of d2-differentiated LUHMES cells to higher nominal

concentrations. We conclude that TEBU shows a strong tendency to adsorb to plastic surfaces and that protein in the medium can markedly reduce these losses, thereby altering the effective exposure (free concentration) in vitro. For hydrophobic compounds like TEBU, plastic binding and protein interactions need to be taken into account, and cellular concentrations may differ substantially from nominal medium concentrations.

P308
Non-human primate gut organoids: A translational in vitro platform for gastrointestinal drug safety testing
ABSTRACT #362

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Background and Objectives: Gastrointestinal (GI) toxicity is a frequent adverse finding in nonhuman primate (NHP) in vivo drug safety studies and often limits the progression of otherwise promising drug candidates into clinical development. While human intestinal organoid systems are widely used to investigate mechanisms of drug-induced GI injury, a critical translational gap remains: no well-established NHP gut organoid models exist, capable of mechanistically interrogating GI toxicities observed in NHPs in vivo. Here, we report the development and comprehensive characterization of region-specific gut organoids derived from primary intestinal tissues of the cynomolgus macaque (Macaca fascicularis). Materials and Methods: Organoids were successfully established from the duodenum, jejunum, ileum, cecum, and colon, with optimized culture conditions enabling robust derivation, long-term expansion, and stable propagation across multiple passages. Results: Cynomolgus gut organoids exhibited morphological and architectural features comparable to human intestinal organoids, including crypt-like structures and organized epithelial layers, with an average diameter of ~100 µm and an area of ~8,000 µm². Most organoids displayed correct basal-out polarity, confirmed by apical (ezrin) and basal (integrin β1) marker localization, a prerequisite for functional intestinal epithelial models. Molecular characterization confirmed the expression of stem and proliferation markers, including Lgr5 and additional lineage-specific markers, supporting maintenance of stemness and epithelial identity. Single-cell RNA sequencing further resolved cellular sub-populations within the organoids, demonstrating the presence of key intestinal epithelial lineages. Functional differentiation assays validated the capacity of these organoids to generate region-appropriate intestinal cell types. Discussion and conclusion: Finally, selected drug candidates previously associated with GI toxicity in NHP in vivo studies elicited reproducible and mechanistically informative responses in vitro, demonstrating the translational relevance of this platform. Collectively, this work establishes region-specific cynomolgus macaque gut organoids as a novel NHP in vitro platform, bridging a critical gap between in vivo NHP findings and mechanistic drug safety evaluation.

P309
Human-relevant in vitro approaches to assess gastrointestinal toxicity of food-grade titanium dioxide (E171)
ABSTRACT #431

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Background and Objectives: Food grade titanium dioxide (E171) has been widely used as a whitening agent in foods and pharmaceuticals, resulting in chronic dietary exposure. In vitro and animal studies have suggested that E171 may induce oxidative stress, DNA damage, epithelial dysregulation, and microbiome alterations, but the human relevance of these findings is unclear. As regulatory positions on E171 differ globally, translational evidence is essential to support human relevant risk assessment. Material and Methods: We applied a tiered approach integrating advanced human relevant in vitro systems with a controlled human dietary intervention. Human induced pluripotent stem

cell (iPSC) derived 3D colon organoids were exposed to food grade E171, assessing ROS formation, oxidative DNA damage, and transcriptomic modulation. In parallel, healthy volunteers participated in a cross over intervention receiving a human relevant dose of E171 (2 mg/kg body weight/day) in a realistic food matrix. Systemic oxidative stress markers were quantified in blood and plasma, and transcriptomic profiling was conducted on rectal biopsy samples. Results: In iPSC derived colon organoids, E171 induced dose dependent ROS generation and oxidative DNA damage without cytotoxicity, accompanied by differential expression of pathways related to oxidative stress responses, inflammation, extracellular matrix remodeling, metabolic alterations, and colorectal cancer associated signaling. In the human study, systemic oxidative stress markers showed only limited and non conclusive changes. However, rectal biopsies displayed consistent transcriptional modulation, including activation of oxidative stress related carcinogenesis pathways, PPAR signaling, and metabolic reprogramming, mirroring key molecular responses observed in vitro. Discussion and Conclusion: This work provides the first direct comparison between human colon tissue responses and mechanisms identified in an advanced in vitro colon model exposed to E171. The convergence of transcriptomic alterations highlights the value of human relevant in vitro approaches for gastrointestinal toxicity assessment and supports their integration into next generation risk evaluation frameworks.

P310
Impact of dose metric selection on transcriptomic point of departure (tPOD) derivation in in vitro toxicology ABSTRACT #439

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Modern toxicological assessments increasingly use in vitro assays for prioritization and potency-based ranking, yet nominal concentrations may not reflect biologically relevant exposure. Mass balance modelling estimates compound bioavailability accounting for non-specific binding, partitioning, and intracellular distribution under steady-state assumptions. This study examines how dose metric selection influences outcomes when applying mass balance modelling to tPoDs derived from in vitro assays, used for agrochemical research compounds. High-throughput transcriptomic profiling used commercially available human cell lines and DRUG-seq methodology. Cells were exposed to 20 research agrochemical compounds for 24 hours across an eight-point dose–response curve (≤100µM; half-log dilutions; n=5). Benchmark dose (BMD) modelling was conducted using BMDExpress3. Gene-level BMDs were calculated from modelled concentrations using a Williams Trend Test prefilter (FDR < 0.05; fold change > 1.5), followed by BMD estimation using polynomial, exponential, and Hill models (benchmark response > 1 SD). Compound level tPoDs were the median BMD of the lowest significantly enriched GO Biological Process term (FDR <0.05; 5–100 genes; BMDL/BMDU <40). Compound distribution was predicted using the mass balance model described by Armitage et al. (2014, 2021). BMD calculations were generated from both nominal and mass balance–adjusted concentrations, applied post hoc or prior to modelling. Chemical ranking varied markedly with dose metric choice. Divergent physicochemical properties drove these differences: high logP increased non-specific binding yet enhanced uptake; low solubility reduced free and intracellular concentrations; and pKa/pKb influenced cellular localisation and uptake. Consequences included reinterpretation of low potency responses after correcting for bioavailable concentrations, divergent rankings depending on when corrections were applied, and changes in ranking when using free or intracellular rather than nominal concentrations. Dose metric selection critically influences tPoD derivation and potency-based ranking. While distribution modelling supports prediction of compound behaviour in vitro, careful metric selection and associated uncertainties are essential for robust screening and decision-making.

P352
Identification of biotransformation products of amitraz in SH-SY5Y spheroids using LC-Q-TOF MS/MS

ABSTRACT #465

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Amitraz (AMZ) is a formamidine acaricide widely used in apiculture to control Varroa destructor, a major threat to honey bee colonies. Due to its extensive use in beekeeping, residues of AMZ and metabolites have been detected in honey and other beehive-derived products, raising concerns about dietary exposure. AMZ is chemically unstable and can generate metabolites such as N-(2,4-dimethylphenyl)-N′-methylformamidine (DMF) and 2,4-dimethylaniline (DMA), which may contribute to its toxicological profile. AMZ has been associated with neurotoxic effects, including central nervous system depression, overexcitation and autonomic dysfunction, mainly related to its interaction with α2-adrenergic signaling. Therefore, characterizing AMZ biotransformation products in neuron-relevant models is essential to better understand its toxicological relevance. In this study, the time-dependent distribution and biotransformation of AMZ were investigated in SH-SY5Y spheroids, a neural-like in vitro model. Although SH-SY5Y cells are expected to display lower xenobiotic-metabolizing capacity than hepatic models such as HepG2, their use enables the assessment of AMZ uptake, intracellular fate and metabolite formation in a microenvironment closer to neural tissue. Spheroids were exposed to AMZ and sampled at 0, 0.5, 1, 2, 4, 8 and 24 hours. Culture supernatants and isolated spheroids were collected, and AMZ, DMF and DMA levels were determined by LC-Q-TOF MS/MS. AMZ levels rapidly decreased in the culture supernatant during the first hours of exposure, with transient accumulation in the spheroid fraction. DMF and DMA were detected from early time points, showing a progressive increase over time and higher relative abundance in isolated spheroids than in the supernatant. These findings support the relevance of SH-SY5Y spheroids as a model for studying AMZ biotransformation and the combined toxicological impact of AMZ and its metabolites in neuron-relevant systems. Acknowledgments: This research was funded by MICIU /AEI /10.13039/501100011033 / FEDER, UE through the project PID2024-160527OB-I00. F, Franco-Campos thanks to the scholarship "Santiago Grisolía (CIGRIS/2021/073)".

P353
IMPLENTATION OF A ZEBRAFISH EMBRYO PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR HIGH THROUGHPUT TOXICOLOGICAL RISK ASSESSMENTS
ABSTRACT #470

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Zebrafish hold a unique role in toxicity testing by bridging between in vitro and in vivo models. As a whole organism system, zebrafish capture complex biological processes, such as metabolism, organ-organ interactions, and behavior endpoints, while still allowing for high-throughput assessments. However, translating zebrafish bioactivity values to human exposures to properly inform toxicological assessments remains challenging. While nominal concentration is typically used for hazard evaluation, this may poorly reflect internal concentration due to differences in chemical bioavailability, making extrapolation to humans difficult. Physiologically based pharmacokinetic (PBPK) models allow for prediction of internal concentrations based on simulation of physiological processes. Although several zebrafish PBPK models have been developed, these were limited to small subsets of chemicals. Here, we present a zebrafish embryo PBPK model based on prior work that

was refined using experimental zebrafish and embryo bioavailability data. By integrating bioavailability data generated by our group and literature sources, we extended the model to cover ~300 chemicals spanning a broad range of physicochemical properties. The model captures development over the first 120 hours post fertilization, metabolism, and uncertainty estimated through Bayesian inference. Sensitivity analyses were performed to identify key physicochemical properties and tissue partition coefficients driving chemical pharmacokinetics. We identified the fish whole body biotransformation rate as the most accurate prediction parameter for zebrafish metabolism, alleviating the need to generate experimental metabolic clearance data. Bioavailability data from zebrafish embryos fell within the PBPK modeled prediction intervals for the majority of chemicals. This model provides a tool to facilitate extrapolation of zebrafish toxicity data for human risk assessment. This project was supported by the National Institute of Environmental Health Sciences, National Institutes of Health under contracts HHSN273201500010C (Inotiv, Inc.) and the Division of Program Coordination, Planning, and Strategic Initiatives, National Institutes of Health under contract GS-35F-393CA - 75N94025F00103 (General Dynamics Information Technology).

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