Abstracts of the 12th World Congress on Alternatives and Animal Use in the Life Sciences, Niagara Falls, 2023

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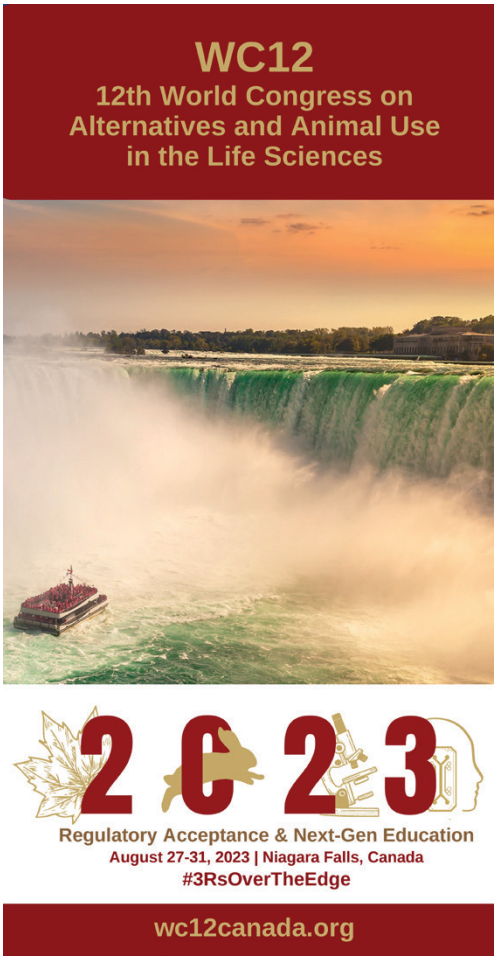

Springer Spektrum

Dear World Congress Community:

On behalf of the Canadian Organizing Committee, it is my honour and privilege to welcome you to the 12th World Congress on Alternatives and Animal Use in the Life Sciences in the majestic city of Niagara Falls, Ontario, Canada. We gratefully acknowledge that WC12 is hosted on the traditional territories of the Anishinaabe and the Haudenosaunee Peoples of Canada.

This year, the World Congress celebrates 30 years of progress in the field. As we gather in this iconic setting where the formidable force of nature mirrors the boundless potential of scientific discovery, we must renew our shared commitment to seek innovative, scientifically robust methods that replace the reliance on animals across the life sciences. Niagara Falls, with its timeless beauty and raw power, also serves as a poignant reminder of the inter-connectedness of all life on our planet. Let us renew our collective dedication to higher ethical standards to move beyond the 3Rs – #3RsOverTheEdge – to protect animals in science.

Over the past decade, our field has witnessed remarkable advancements in new approach methodologies (NAMs), innovatively designed to supplant traditional animal testing. However, widespread integration of these NAMs into regulatory risk assessment remains limited to specific assays and selective nations. Our challenges – both pressing and profound in nature – will require us to seize the untapped potential inherent in NAMs and seamlessly integrate them into the global framework of hypothesis-driven biomedical research, drug development pipeline, chemical safety testing, and beyond into the biology classroom. The holistic realization of this 21st-century vision, in turn, necessitates the conscientious cultivation of the next generation – instilling within them the torch-bearing responsibility to carry forth the pioneering progress made in the field. With these challenges in mind while developing the WC12 program, we aspired to facilitate and foster a platform with diverse themes to champion the exchange of ideas, exploration of differing viewpoints, and forging of consensus among all stakeholders to advance 21st-century science, innovation, and ethics.

We would like to extend our deepest gratitude to everyone who contributed to the success of WC12! May WC12 serve as a beacon of hope and inspiration, illuminating an accelerated path towards a future where innovative and ethical practices redefine the landscape of scientific research and regulatory testing to protect humans, animals, and our environment!

On behalf of the Canadian Organizing Committee,

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WC12 Chair
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Canadian Centre for Alternatives to Animal Methods (CCAAM)
Canadian Centre for the Validation of Alternative Methods (CaCVAM)

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ROBERT KAVLOCK
Former Acting Assistant Administrator, US Environmental Protection Agency

ACT BOARD MEMBER

Welcome to WC12 from the Alternative Congress Trust

As we gather for the 12th World Congress on Alternatives and Animal Use in the Life Sciences (WC12), we are not only embracing a tradition of scientific excellence but also celebrating a milestone - the 30th anniversary of these vital conferences. From the very first assembly in Baltimore initiated by the Johns Hopkins Center for Alternatives to Animal Testing (CAAT), these congresses have stood as a beacon, guiding the path towards ethical, innovative, and sustainable scientific practice.

The progress made over the last three decades has been remarkable. The themes for WC12 of Transparency, Collaboration, Innovation, and Transformation represent the core values that have guided this movement since its inception. These principles continue to foster interdisciplinary cooperation, driving forward cutting-edge methods that seek to minimize, refine, and replace animal usage in the life sciences.

This congress' significance extends beyond mere celebration. It offers an opportunity to reflect upon the journey that has led us here and to envision the path ahead. Our collective efforts have led to incredible advancements in alternatives to animal testing, such as microphysiological systems, *in vitro* models, and computational tools. It is a journey marked by collaboration, not only between different scientific

disciplines but between academia, industry, governmental bodies, and NGOs. We are honored to have eminent scientists, policymakers, and researchers among us as a testament to the innovative spirit of this community.

Within these pages, you will find abstracts that represent the latest in cutting-edge research, policy development, and ethical considerations. From the complexities of microphysiological systems to refinements in animal research practices, the scope and depth of the content reflect the evolving and dynamic nature of this field.

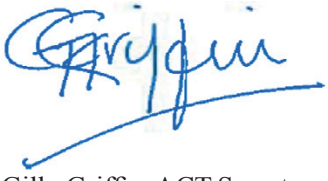
As we look to the future, we see a horizon filled with potential. The knowledge, creativity, and compassion that have marked the past 30 years will continue to guide us as we strive for a world where ethical scientific practice is not just an ideal, but a reality.

On behalf of the Alternative Congress Trust, responsible for the World Congress series, we extend our warmest welcome to all participants. May this year's gathering be a platform for inspiration, reflection, and renewed commitment to the principles that have shaped 30 years of progress.

Wishing you an enlightening and collaborative experience at WC12.

Elaine Faustman, ACT Chair

Thomas Hartung, ACT Treasurer



Gilly Griffin, ACT Secretary

Dear WC12 participants,

ALTEX Proceedings is proud to present the Abstract Book of the 12th World Congress on Alternatives and Animal Use in the Life Sciences in Niagara Falls, Canada.

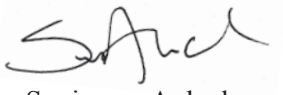
This Abstract Book contains summaries of about 350 oral presentations and 330 posters. They are sorted by presentation type and submission ID number. The abstracts address the six Congress Themes: Regulatory Acceptance and Global Harmonization; Next-Gen Education; Ethics, Welfare, Policies, and Regulations; Human-Centered Biomedical Research; Refinement and Impact on Science; 21st Century Predictive Toxicology. They represent the work of more than 2200 contributing authors from 40 countries on all six continents.

We are very grateful to the Doerenkamp-Zbinden Foundation, Switzerland for again generously funding the production of the Abstract Book. Thank you to Melanie Gubbels-Schols from Klinkhamer Group for her cooperation in producing the Abstract Book.

Two years after WC11 was held as a virtual congress, we are all thrilled to be meeting face to face again in this majestic location. We wish all participants of WC12 an inspiring meeting that will bring forth new ideas and international collaborations in the effort to replace, reduce, and refine animal experiments towards human-relevant and humane science.

We can now already start looking forward to WC13, which will bring us together again in Rio de Janeiro, Brazil in 2025.

With best wishes,



Sonja von Aulock
Editor in chief, ALTEX & ALTEX Proceedings

#UseScienceNotAnimals

Unilever manufactures a range of home, personal care and food products that are sold throughout the world in over 190 countries, with over 400 brand names. On any given day, 3.4 billion consumers use Unilever products sold under many well-known brands, including Dove, Lifebuoy, Comfort, Sunlight, Axe and Knorr.

SEAC (Unilever's Safety and Environmental Assurance Centre - seac.unilever.com) is a key part of Unilever R&D with responsibility for ensuring that Unilever's innovations are designed to be safe and sustainable. Our leading-edge approach to assuring the safety of Unilever's products for people and the planet has one clear purpose: to guarantee that our products are safe, without the need for animal testing. That's why we say #UseScienceNotAnimals.

Our ability to innovate using non-animal safety assessment approaches is underpinned by scientific partnerships with over 70 leading research teams globally to develop and apply new capability. We look to openly share the experience gained from these collaborations through publications, presentations and through our website.

Unilever have supported World Congress events for over 25 years, and we look forward to WC12 and the opportunity to explore how we can accelerate Regulatory Acceptance and Next-Gen Education together! This year we are proudly sponsoring the #UseScienceNotAnimals World Café as well as presenting many aspects of our collaborative research through the scientific program. Stop by our WC12 booth or head to our website to find out more: **https://seac.unilever.com/wc12/**

Our Efforts to End Animal Testing

$480 million+
of investment

40 years
of commitment

25+
non-animal research methods pioneered

Partnering with leading international animal welfare organisations, academia, and industry coalitions

Advocating for public and scientific use of non-animal methods to policy makers world-wide for over 25 years

Our Commitment to #BeCrueltyFree

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

Our Animal Welfare Achievements

*Ending Animal Testing – **Together***

Oral Presentations

1

Why I moved from working on refinement to replacement

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As a newly graduated vet I wanted to make a difference for laboratory animals through committing myself to research and education on the implementation of the 3Rs. At that time, I still believed that animal studies were needed, and I started my career focusing mainly on refinement and reduction. After decades of experience in the laboratory animal science field, it became clear that focusing on the 3Rs is in itself not a successful way towards 3R implementation. Therefore, I started working on preclinical systematic reviews, as this methodology seemed the way forward: results from systematic reviews can lead to direct 3R implementation and a lot more. However, the unfortunate benefit of systematic reviews is that the results have demonstrated again and again that many publications on animal studies lack essential details and that the translatability of animal studies to humans is often very low. Moreover, a historical analysis of the regulatory requirements on animal studies has revealed that these requirements are often not based on sound scientific evidence. The focus on refinement and reduction can also prevent focusing on replacement. The results of new approach methods have indicated that translation to the human situation can be much higher and thus more promising. It is therefore high time to move the prime focus to replacement and accelerate the transition to animal-testing-free science and regulations, for the benefit of animals and humans.

Presentation: Oral

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Animal-reliance bias mitigation: Developing evidence from transdisciplinary research

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Animal-reliance bias refers to a reliance on or preference for animal-based methods where they may not be necessary or where non-animal-based methods may be (more) suitable. This bias affects the likelihood of a manuscript begin accepted for publication and of a project application being rewarded for funding. Currently, there are clear indications that this bias exists in the publishing and funding process, favoring animal research as this is generally still considered the gold standard. However, what is the evidence telling us? Evidence from systematic reviews – which are by nature an interdisciplinary endeavor – has shown repeatedly that the publication quality of animal studies is often insufficient and hardly improves over time, and that many animal studies do not translate well to the human situation. The poor publication quality makes it impossible to reliably interpret the research results, and additionally also poses an ethical problem concerning the use of animals. Because many animal studies are done with the aim to improve human health, the poor translation of animal studies leads to the important question whether it is worthwhile to continue doing animal studies for that purpose. Therefore, it is useful to address research questions from a more holistic perspective by doing transdisciplinary research. Transdisciplinary research integrates knowledge across academic disciplines (interdisciplinary) and with non-academic stakeholders to address societal challenges. In my presentation I will give examples of how transdisciplinary research and especially transition science can be beneficial, connecting different fields of research, stakeholders, and society.

Presentation: Oral

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Animal-free recombinant antibodies for research and diagnostics

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While phage display, the premier animal-free method for antibody production, is well established for the production of therapeutics, most antibodies for research and diagnostics are still produced in animals. This presentation will review the achievements and prospects of recombinant *in vitro* antibody generation and provide examples of how animal-derived antibodies could be supplemented or replaced in typical current research applications. Further differences and opportunities of *in vitro* antibody generation compared to animal-based generation will be presented, e.g., the possibility to predetermine specificity features, the exchange of constant regions to adapt to different detection antibodies (species switch), or easy post-selection modifications to add functions not available from plain IgG. In addition, polyclonal animal antibodies can now be replaced by recombinant antibodies in various applications, from research and diagnostics to passive vaccinations.

Presentation: Oral

11

Goals for integrating NAMs into next generation education

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The conduct of unnecessary animal tests that are not justifiable from a scientific or ethical perspective needs to be avoided and modern non-animal science and New Approach Methods (NAM) need to be implemented. How can we make the change towards the fast integration of NAMs in education? The goals are to educate scientists and regulators to close the gaps in knowledge and application, and to also improve the confidence in NAMs. Already many educational tools and methods exist which can be implemented in the short term: Learning scenarios on NAM teaching programs have been made freely available via the JRC website. At the ETPLAS training platform, e-learnings on searching for and application of NAMs have been made freely available (52 and 60). At Utrecht University we have invited industry to take part in education on the next generation risk assessment (NGRA). The students valued this very much. Also, co-creative interdisciplinary learning of students and teachers can be implemented immediately and can be a very productive method in making interdisciplinary progress together fast (Abarkan, 2022). In the longer term, new learning scenarios and roadmaps for education will help to make further changes and commitments towards non-animal science. In the Netherlands the university organizations UNL and NFU have, e.g., committed themselves to a target image to move to the implementation of animal-free methods in education. That way animal use in teaching is avoided and moreover, influencing the thinking into the possibilities of non-animal education and research.

Presentation: Oral

12

Animal methods bias in NIH research funding review committees

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There is broad agreement among researchers, policy-makers, and regulators that increasing the use and awareness of non-animal research methods is needed to reduce the numbers of animals used in science. However, progress in this area has been slow and animals continue to be used even in research areas where non-animal methods are available. Our research will attempt to ascertain whether a greater familiarity with animal-based methods within research project review committees may contribute to the continued use of animals in biomedical research in the U.S. To investigate this, People for the Ethical Treatment of Animals is conducting an analysis of the types of research methodology expertise held by members of U.S. National Institutes of Health Center for Scientific Review study section members. Using the agency's "iCite" tool, we will assess the extent to which individual committee members' publication profiles are oriented towards human, animal, and cellular/molecular research. Preliminary data suggest NIH study sections evaluating basic and translational research proposals are disproportionately composed of reviewers with primary expertise in animal-based methods, and animal use among reviewers positively correlates with the number of animal-based grants funded. The implication of these data is that review bodies without sufficient expertise in non-animal methods may not be providing fair review and consideration to research proposals that propose to use non-animal methods. We expect this research to demonstrate the necessity for systemic and cultural change in the biomedical research community and be used to advocate for polices that raise the bar on ethical and effective research.

Presentation: Oral

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Global trends in laboratory primate use: 1950 - present

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The laboratory animal supply industry is relatively young. For example, when Eli Lilly needed rabbits to standardize the different batches of insulin produced to treat diabetics, one of the big challenges they faced in the late 1920s was sourcing sufficient numbers of white rabbits for the bioassays. A similar challenge arose in the period immediately after World War II when rhesus monkeys became the animal of choice in which to grow the polio virus stocks from which the Sabin vaccine was manufactured. The presentation will track global laboratory primate use over the past 75 years and identify the reasons for changes in such use and how alternative methods have reduced the demand for live monkeys.

Presentation: Oral

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Protocol review and the promotion of alternatives

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Institutional Ethics and Animal Care Committees have been criticized for not rejecting a greater number of the proposed research and testing protocols submitted by faculty and research staff for approval. Such criticism misunderstands the role, actual and potential, that these committees provide in their institutions. The committee members are mostly colleagues of those submitting protocols for review and, as such, they are expected to engage in constructive dialogue with their colleagues. Such dialogue more usually results in the modification of protocols, not their absolute rejection. Several specific (but anonymous) examples of such dialogues taken from the author's own experience on protocol review committees will be provided.

Presentation: Oral

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International computational collaborations for predictive toxicology

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Humans are exposed to an increasing number of chemicals, but only a fraction of these have been evaluated for potential risks to human health and the environment. Thus, both regulators and manufacturers need rapid and efficient approaches to evaluate the potential toxicity of thousands of chemicals already in commerce and others in development. Advances in information technology and machine learning have fostered the development of *in silico* approaches that leverage the relationships between chemical structures and their biological activities. However, individual predictive computational tools are associated with certain limitations, and they are only as good as the input data upon which they are built. To address these challenges, international consortia involving over 100 scientists from governmental agencies, academia, and industry collaboratively developed *in silico* tools for predicting chemical toxicity based on mined and curated data from the literature. These consortia have successfully concluded three projects: the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP), the Collaborative Modeling Project for Androgen Receptor Activity (CoMPARA), and the Collaborative Acute Toxicity Modeling Suite (CATMoS). Limitations of individual modeling approaches were overcome by establishing consensus models that leveraged each model's strengths. The resulting consensus models have been used to screen hundreds of thousands of chemicals from the U.S. Environmental Protection Agency's (EPA's) DSSTox database. These models are freely available for further use through the open-source suite of QSAR models OPERA, as an installed standalone application, by querying the EPA's CompTox chemistry Dashboard and NTP's Integrated Chemical Environment, or within the OECD toolbox through OPERA's plugin.

Presentation: Oral

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Acute toxicity *in silico* models and expert reviews

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This presentation will outline modern developments in *in silico* alternative approaches that leverage the wealth of historical data generated through machine learning methods. It will describe the development of a battery of transparent and fit-for-purpose *in silico* models to evaluate the acute toxicity 6-pack: (1) acute systemic toxicity by three routes of exposure (oral, dermal, inhalation), (2) skin and eye irritation/corrosion, and (3) skin sensitization. To support the development of these models, a series of databases were constructed including hundreds of thousands of chemicals with data supporting these endpoints. The models predict categories such as the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) categories. Two computational methodologies (expert rule-based and statistical-based) across all 6 endpoints and will be presented. The poster will review the development and performance of these models and present workflows to illustrate how they can be used to support the 3Rs, including how an expert review can be incorporated into any assessment.

Presentation: Oral

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Introduction to PBPK modeling for chemical risk assessment

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As risk assessment is shifting from animal testing to new approaches methods (NAMs) based on *in vitro* and *in silico* methods, alternative strategies are needed to accurately integrate and use this non-animal data. Next Generation Risk Assessment (NGRA) of a chemical integrates NAMs to assure human safety without performing animal testing. The key principle is to extrapolate *in vitro* points of departure to external bioequivalent exposures. PBPK modeling is an efficient and reliable tool to simulate the fate of chemicals within an organism and is constructed using a series of differential equations that are parameterized with physiological and chemical-specific variables. Used in combination with state-of-the-art quantitative structure-activity relationship (QSAR), read-across, and *in vitro* testing technologies,

PBPK modeling serves as a tool to predict tissue doses of chemicals and assist in assessing safety of chemicals. The design of a model structure is the first step of model development, and it depends on physicochemical properties, mode of action, and available datasets of the chemical, as well as the purpose of the model. PBPK models need to be carefully evaluated before being used confidently in decision-making context. The Organization of Economic Cooperation and Development (OECD) recently published a harmonized guidance on model application in risk assessment and good modelling practices. This guidance document focuses on parameterizing and validating PBPK models when *in vivo* kinetic data are limited or not available, especially for use in risk assessment. The specific question that the model addresses is essential as it structures model conceptualization, implementation, and evaluation.

Presentation: Oral

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The iron fist and velvet glove: Expanding the implementation of the 3Rs

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While researchers in principle may support the expansion of the 3Rs, they are not necessarily proactive in implementing them for their own research. This is, however, a role that the animal care and use program (ACUP) and especially the IACUC may and should play. This presentation offers a discussion of how the ACUP and IACUC may push researchers to further replace, reduce, and refine their use of animals using both the Velvet Glove (education, support, encouragement) and the Iron Fist (regulatory action) approaches.

Presentation: Oral

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The electro-mitochondrial coupling of a microphysiological human heart

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Cardiovascular diseases are the leading cause of death worldwide. Efforts to study cardiac dysfunction are frustrated by physiological differences between humans and animal models. Here we present a sensor-embedded, hiPSC-derived model of vascularized cardiac tissue that develops into complex multi-chambered self-paced heart organoid under anisotropic stress. Sensor integration permits the simultaneous real-time measurements of oxygen uptake, extracellular field-potential, and cardiac contraction with > 10-Hz resolution. Using this platform, we discovered 1-Hz cardiac respiratory cycles, whose frequency is coupled to the electrical, rather than the previously theorized mechanical, activity of the tissue. Microscopic analysis revealed that calcium oscillations drive a mitochondrial respiration cycle. Pharmaceutical or genetic inhibition of this electro-mitochondrial coupling leads to arrhythmogenic behavior. We show that the chemotherapeutic mitoxantrone induces arrhythmia by disturbing the electro-mitochondrial coupling and that this effect is partly reversed by co-administration of metformin, suggesting a mitochondrial basis for chemotherapy-induced arrhythmia. Our work describes the mitochondrial dynamics of cardiac rhythms, underscoring the utility of microphysiological systems for advancing our understanding of cardiac physiology and advancing pharmaceutical interventions.

Presentation: Oral

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Language and workflow in biomedical research indicate institutional bias and how translational medicine can help abolish non-human animal experimentation

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Non-human research is favored due to institutionalized bias. Structures and proceedings are pre-established for non-human animal researchers. Studies with human material not relying on non-human animals are chronically underfunded and structurally disadvantaged. While this is caused by multiple factors, the most striking ones are language in publication, research and funding structures and precarious work conditions in academia.

In academic education non-human animals are "tools", "models". The non-human subjects are objectified, and passive voice enhances the distance between research and lab-animal. Structures in institutes and suppliers are specialized in non-human animal research. Laboratories are suited for animal research and this work is plannable and easy to fit into a schedule. Milestones can be reached as promised. Working with human primary material heavily relies on individual dedication and motivation. Infrastructures need to be set up separately and follow no clear flow. Work packages are not easily defined, days can vary when working with the clinic. Surgeries can be postponed, and days can be overly stressful or utterly boring. Non-human animal research is scheduled and rarely changed. There is more help, communication, and possibilities for non-human animal researchers to obtain homogenous, reproducible data. PhDs and postdocs rely on data consistently produced, but often have short-term contracts. Finding a patient cohort and sample acquisition often needs more time. Additional hurdles can slow down or alter a work-plan, making milestones less easy to achieve. Shifting focus towards human-based research is beneficial for everyone involved in biomedicine. Working with patients needs patience but is worth it.

Presentation: Oral

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Helpathons to accelerate human-relevant science

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Background: Animal-free innovation becomes easier if you choose to do it together: with patients, researchers, financiers, health professionals and policy makers. A helpathon, inspired by the Dutch "transition to animal free innovations (TPI)" network, is an innovative 2-day workshop. An interdisciplinary group, guided by the Helpathon Team, assists a scientist to transition to animal free research by sharing resources, knowledge and necessary networks and helps find funding.

Method: Key moments of the helpathon workshop:

Opening Phase: the central research question is illuminated by the scientist.

Open Space Phase: has no predetermined agenda! Everything considered important to the central question is covered, thought ought and disconnected from the animal method.

Try Out Phase: a "Power of imagination" session, in which possible solutions are described as if they exist. Promising ideas are further developed.

Communication Phase: Helpathons end with a festive presentation of results to those involved and interested people from the participants' network.

Follow-up Phase: One month later, check back with scientist to see if additional help from the network is needed. All participants receive follow-up information on what happened to the ideas they generated.

Results and Conclusion: Quote from scientist: "It is now clearer to us that the use of animals is not always necessary. Thanks to this TPI Helpathon we have gained new insights about existing *in vitro* and cell culture systems that we can use to test our therapies. These models can give results that may be more relevant to humans than results obtained from laboratory animals."

Presentation: Oral

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Raising concerns for good animal welfare and reproducible science

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It is important for public confidence in laboratory animal science that the standards we work to are high. Technicians, scientists, inspectors and others may identify a concern which arises in any one of many areas of our work with laboratory animals. For example, the facilities may not be up to standard, numbers of staff are insufficient for the tasks required, procedures are not being performed in the most refined manner, or experimental design can be improved. We can all contribute to improving standards when this occurs.

Methods and ideas for addressing concerns will be presented. Defining the precise issue and its consequences is an important first step. It is crucial to identify accurately who is or could be involved, both in the issue itself and in creating a solution. The timeframe for resolution of the problem needs to be identified. The consequences of failure to resolve it, both in the short and long term will assist in determining priorities and resource allocation.

The person identifying the problem should be involved in the resolution, or at least in the follow up, to make sure the issue is resolved to ensure closure and relevant feedback is provided to add to job satisfaction. Working together constructively to ensure high standards can be restored or attained is critical so that good animal welfare and sound reproducible science is the norm. The key to resolution is team-working, a no-blame culture and longer-term follow-up.

Presentation: Oral

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Oversight to facilitate high-quality training

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Training in laboratory animal science should be a career-spanning. At all stages, oversight can be provided to ensure that the training is up-to-date, sufficiently detailed for the purpose, likely to be understood and learned by the trainee.

Introductory theoretical and skills training can be devised to cover a pre-determined set of learning outcomes covering focused and topics specific for trainees' requirements. The quality of training can be assured by the use of suitably qualified / experienced trainers which is clearly evidenced. Assurance that the material has been understood and training has been successful can be acquired by a robust and transparent assessment system, and certification.

Oversight by accrediting or approving bodies can assure that all the content is covered, that trainers are indeed properly qualified, and the assessment is fair and delivered with proper integrity.

Outcomes of the training will be seen by scientists, technicians and inspectors who should see evidence of an appropriate level of competence and knowledge resulting from training.

The role of the inspectors in overseeing quality of outputs, and in indicating any areas for improvement, along with the likely source of the solution will be explored.

Trust in high-quality training and records of training should allow transferal between institutions with minimal repeated training, and provide a platform for peer-review of quality of laboratory animal science throughout careers to improve the animal welfare and 3Rs.

Presentation: Oral

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Physiologically-based kinetic modelling as fundamental for next generation risk assessment

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Chemical risk assessment for human recently increases the need of using new alternatives methodologies (NAMs), and the concomitant to phase out of animal testing. Next Generation Risk Assessment (NGRA) of a chemical is a framework that integrates NAMs to assure human safety without animal testing. The NGRA requires extrapolations of the *in vitro* points of departure (PoDs) to external equivalent exposure, relevant to specific exposure scenarios. Physiologically based kinetic (PBK) models describe the fate and integrate the knowledge on the absorption, distribution, metabolism, and excretion (ADME) of a chemical in the human body, which provides a means for this extrapolation. The crucial role of PBK modelling in NGRA was demonstrated in a several case studies. These case studies provide guidance in implementing, gaining confidence, and improving regulatory implementation of PBK models for chemical risk assessment. In the presented study, PBK models were developed and qualified for UV filters, e.g., homosalate, to support the human health safety evaluation based on the internal exposure. The aim of this PBK modeling approach is to perform route-to-route and inter-species extrapolations to translate both the oral exposures from a historical rat study, and dermal exposures in consumers using sunscreen to internal dose metrics.

The developed PBK models estimated reasonably well the internal exposures in rats and humans. This helped replace default uncertainty factors with more chemical-specific risk assessment. In the absence of *in vivo* data, such human PBK models will be the key of future non-animal-based risk assessments.

Presentation: Oral

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Mechanistic computational modeling for chemical toxicity evaluations

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Addressing the safety aspects of new chemicals has historically been undertaken through animal testing studies, which are expensive and time-consuming. Due to the massive available data for known chemicals, modern computational toxicology has been advanced to the "big data" era. Central to this shift is the development of machine learning approaches to implementing innovative modeling based on the dynamic, heterogeneous, and large nature of public chemical toxicity data sets.

Our recently developed computational approaches and relevant modeling studies answered above challenge by providing new solutions to chemical toxicity evaluations based on data-driven mechanistic modeling. For example, we developed predictive machine learning and deep learning models for animal acute toxicity, developmental toxicity, and human hepatotoxicity using both chemical structure information and public big data. One of these studies was to use a knowledgebase deep neural network (k-DNN) approach to simulate the adverse outcome pathways that can induce rodent uterotrophic bioactivity, The resulted computational toxicity models can predict the rodent uterotrophic bioactivity through illustrating toxicity mechanisms by constructing various adverse outcome pathways for all predicted toxicants. To strengthen the model predictions, we implement new modeling approaches that can incorporate dose dependent toxicity responses and toxicokinetics data (e.g. chemical exposure plasma concentrations) into the chemical risk assessments.

The big data mining, analysis, and modeling techniques in these studies advanced artificial intelligence in the big data era, which has paved the road to future computational chemical toxicology and will have a significant impact on the risk assessment procedure and public health.

Presentation: Oral

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Introduction to the traditional concept of *in vivo* adversity

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Toxicity testing is undergoing a remarkable paradigm shift brought about by the advent of new approaches to understanding cellular responses to toxic exposures. This paradigm shift was articulated by the 2007 National Academy of Sciences report titled "Toxicity Testing in the 21st Century: A Vision and a Strategy," and a world-wide effort is underway to define the tools and interpretative frameworks needed for this new paradigm to succeed.

One of the challenges of this new strategy is how to understand and identify adversity within the predominantly cell-based platforms used in the new test systems. To address this challenge, it is helpful to look back on how adversity is defined in animals used for toxicity testing. A common definition of organismal adversity is that proposed by R. W. Lewis et al., 2002:

"Adverse effect – A biochemical, morphological or physiological change (in response to a stimulus) that either singly or in combination adversely affects the performance of the whole organism or reduces the organism's ability to respond to an additional environmental challenge."

In practice, most adverse effects identified in animal studies are those that produce statistically significant changes in an endpoint, such as body or organ weight, and clinical chemistry or histopathological alterations commonly accepted by pathologists as indicative of a non-adaptive or irreversible change in an organ system.

This session explores how *in vivo* adversity based on changes in whole animal endpoints can be adapted to identify actionable alterations in structure and function of cell-based systems that allow a safety decision.

Presentation: Oral

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Patient biomimetic twins in precision medicine

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The heterogeneity of patient genetics, environment, and lifestyle have made it challenging for creating safe and efficacious therapeutics for many common, chronic disorders. Also, experimental animal models do not always recapitulate human disease and raise significant ethical and regulatory concerns. To address these challenges, we have created patient-specific liver microphysiological systems (MPS) produced from primary human cells and patient-derived induced pluripotent stem cells (iPSCs) that are then differentiated into hepatocytes, endothelial cells, stellate and Kupfer cells. We have created a 250-patient cohort with clinical phenotyping and linked to biospecimens for generating multimodal datasets to create "patient digital twins (PDTs)". We have also generated patient-specific liver MPS from the same patients, "patient biomimetic twins (PBTs)". We have recapitulated metabolic-dysfunction associated fatty liver disease (MAFLD) over a two-week period. We reproduce the patient environment and dietary and metabolic pattern based on the clinomics data and define the glucose, insulin, free fatty acids (FFA) and immune activation molecules found in each patient for the media used in their PBT. We have quantified the level of steatosis and other metabolic dysfunctions, immune activation, and fibrosis in the PBTs with a variety of imaging, metabolomic, genomic and efflux media analyses. We are developing a semi-automated platform for incubation, fluidic control, imaging, and efflux media sampling under the control of an analytics and database tool (Biosystics-Analytics Platform™). This integrated platform will be useful in investigating mechanisms of disease progression, toxicology, drug discovery and optimized clinical trials for any disease MPS and coupled organ MPS.

Presentation: Oral

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Artificial intelligence for regulatory science research

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Artificial Intelligence (AI) is a broad concept of training machines to think and behave like humans. It consists of a wide range of statistical and machinal leaning approaches to learn from the existing data/information to predict future outcomes. It has impacted a board range of scientific disciplines that are important to public health, including toxicology. The rise of AI has also offered both opportunities and challenges to regulatory agencies with questions such as (1) how to assess and evaluate AI-based products and (2) how to develop and implement AI-based application to improve the agencies functions. In this presentation, the current thinking and on-going efforts in the area of AI for risk assessment will be discussed with examples from FDA projects. The guiding principle and best practice of applying AI in regulatory science research will also be discussed with respect to the context of use and fit-for-purpose application.

Presentation: Oral

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Applying the RSPCA Roadmap to reducing severe suffering in practice – Case studies from a pharmaceutical company

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The use of animals in pharmaceutical research and development is a concern for Novo Nordisk, as it is for the public. Our research focuses on developing treatments for chronic conditions such as diabetes and hemophilia, involving animal models. We are committed to being open about animal use and working with a range of stakeholders to identify ways to minimize harms to animals and improve welfare [1].

We work on implementing all 3Rs and have successfully adapted the step-by-step process of the RSPCA "Roadmap" to reduce severe suffering in different research areas. The presentation will describe how the Roadmap was prospectively applied to three different types of animal model, within toxicology, a disease model and a surgical model. It will also include a summarized outline of

a retrospective, post study application of the Roadmap. These case studies will demonstrate the wide applicability of the Roadmap as a refinement and analysis tool, whether it is used in a pharmaceutical company or academia.

The presentation will also explain how the Roadmap's requirement for a team approach to reducing and avoiding severe suffering facilitates open, constructive dialogue between scientists and animal technologists and care staff. This leads to better understanding and respect between people holding different roles within an establishment, which supports the Culture of Care and facilitates all 3Rs.

Reference

[1] https://novonordisk.com/science-and-technology/bioethics/animal-ethics.html

Presentation: Oral

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Preregistration in animal research – Critical evaluation of the current state

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Before planning an animal study, scientists should thoroughly evaluate the present state of research by a systematic review of the current literature. If they conclude that animal experiments are still necessary to answer a crucial question, maximizing the knowledge gained out of these experiments should be of highest priority. Preregistration can effectively improve the validity and transparency of research and should become the norm in animal research. Preregistration is mandatory for most clinical trials and widely accepted in other disciplines like psychology.

Three registries already encourage the preregistration of animal research, i.e., preclinicaltrials.eu, open science framework registry and animalstudyregistry.org. These registries have now established eight common standards to guarantee the quality of preregistration for animal research. Registries should ensure public accessibility, transparency in their financial sources, tracking of changes, and warranty and sustainability of data (Heinl et al., 2022, *PNAS Nexus 1(1)*). Stakeholders recommending preregistration can now refer to these published standards.

The infrastructure for preregistration in animal research was established in the last years but we have now to critically evaluate why animal scientist are still hesitant about preregistration. It will therefore be crucial to analyze current barriers and to demonstrate the effectivity of preregistration for the research quality. Beyond informing researchers about the possibility of preregistration for animal science and the benefits for their research, we have to convince stakeholders to incentivize preregistration. Contributing to transparency has to become a value of its own, which needs to be rewarded by research institutions, funders, publishers, and regulators.

Presentation: Oral

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Advanced immunocompetent *in vitro* primary human lung models for toxicity assessment and infectious disease research

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The main function of the human airway epithelium is to generate sterile atmosphere for the alveolar region where the gas exchange occurs. As first line of defense against airborne pathogens or xenobiotics, the airway epithelium acts not only as key physical barrier endowed with mucociliary clearance and innate host defense mechanisms, but also as an important immunoregulator through production of key messengers and physical interactions with immune cells especially dendritic cells and macrophages.

We will describe the development and characterization, as well as the use of fully primary human cell-based co-culture models made of nasal, tracheal, bronchial, small-airways and alveolar epithelia (MucilAir™, SmallAir™ and AlveolAir™) and dendritic or alveolar macrophages.

Several applications of these advanced immunocompetent ALI models will be discussed: (i) inhalation toxicity assessment with highlight on OECD Case study 367; (ii) screening of antiviral drugs against SARS-CoV-2, rhinoviruses, influenza, RSV and flue; (iii) host-pathogen interactions in co-culture models of upper or lower airway Epithelium & Alveolar Macrophages using bacteria (*Pseudomonas aeruginosa*, *Streptococcus pneumonia* and *Staphylococcus aureus*) in context of new antibiotic development.

Presentation: Oral

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A "Roadmap" approach to help end "severe" suffering

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Any level of laboratory animal suffering is a concern for the scientific community and public, and reducing and avoiding "severe" suffering (Canadian Category D/E; USA Category E) should be a top priority. The "Focus on Severe Suffering" project – supported by scientists, animal technologists, regulators and lab animal veterinarians – takes a strategic approach to this important animal welfare and ethical issue.

The project initially identified three potential causes of "severe" suffering: (i) inherently severe procedures, e.g., some sepsis models, (ii) mortality and (iii) "cumulative" effects that can become severe. The next step was developing a practical "Roadmap" exercise to enable institutions to critically review procedures that could cause "severe" suffering, identify contributing factors and find ways of avoiding or refining these.

A key principle of the Roadmap [1] is an "audit" of procedures, by an in-house team of people with different expertise and perspectives. This involves reviewing the animal's lifetime experiences, identifying every source of potential suffering and implementing refinement for each one. Comprehensive guidance is available online and users report that they have successfully employed the Roadmap to reduce and avoid severe suffering. This has helped achieve an overall 61% reduction in "severe" experimental procedures in the UK between 2014 and 2020 [2], and its strategic approach can be applied globally. The presentation will explain how the Roadmap works, and how to find and use the online resources, enabling participants to implement practical refinements at their own institutions.

References

[1] focusonseveresuffering.co.uk/roadmap
[2] Animals in science statistics – GOV.UK

Presentation: Oral

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Accelerating the transition to animal-free NGRA: A transformative governance approach

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The aim of this research project is to contribute to the acceleration of the transition to animal-free safety assessment for chemicals and pharmaceuticals in the EU and the USA by applying a transformative governance approach. The project will contribute to solving the problem of low acceptance and implementation of animal-free safety assessment, leading to more competence and trust. The project encompasses the work of 2 PhD students and one postdoc embedded in a large consortium of stakeholders covering "all" scientific fields and stakeholder levels, such as academia, industry, NGOs, and regulatory bodies.

Transformative governance is focused on the underlying causes of societal problems and incorporates three levels of transitions. The project operationalizes these niche, regime and landscape levels as follows. At the niche level, it develops the transdisciplinary knowledge needed to demonstrate the usability and applicability of Next Generation Risk Assessment (NGRA) for chemicals and pharmaceuticals. At the regime level, it facilitates the (regulatory) acceptance of NGRA for chemicals and pharmaceuticals and draws broader lessons for the transition to animal-free safety assessment. At the landscape level, the project analyzes the societal underlying causes of the lack of progress, with a focus on the values, convictions and interests of different societal groups. Envisioned scientific breakthroughs are that evidence is provided that NGRA represents a better scientific approach to safety assessment than animal studies and will be accepted and implemented at the regulatory level. In our presentation the scientific plans and first results will be presented.

Presentation: Oral

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Transcriptomic biomarker validation efforts: Lessons learned from a decade of research on the TGx-DDI biomarker for detecting DNA damaging agents

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Establishing acceptable approaches for the use of *in vitro* transcriptomics in regulatory evaluation is critical to eliminating animal testing. Transcriptomic biomarkers have emerged as objective tools to efficiently analyze and interpret complex gene expression profiles. The TGx-DDI biomarker is an extensively validated and widely used transcriptomic biomarker. TGx-DDI distinguishes DNA damage-inducing (DDI) from non-DDI chemicals based on changes in the expression of 64 biomarker genes in human cells in culture. The biomarker's proposed contexts of use include mode of action analyses, integrated testing and assessment, and chemical screening and prioritization. Efforts to validate and promote regulatory adoption of TGx-DDI have been championed by the nonprofit Health and Environmental Sciences Institute (HESI) Emerging Systems Toxicology in the Assessment of Risk (eSTAR) Committee for over a decade. Extensive validation work (> 100 chemicals) established TGx-DDI applicability across a variety of transcriptomic technologies in TK6 and HepaRG cells. Case studies have demonstrated utility in different decision-making contexts. The eSTAR Committee recognized that a significant challenge to industry and regulatory agencies is interpretation of positive findings for *in vitro* chromosome damage assays when compounds produce a negative Ames and *in vivo* micronucleus tests. The Committee received endorsement via the US Food and Drug Administration (FDA) Biomarker Qualification Program to qualify TGx-DDI as an optional exploratory assay to de-risk compounds that exhibit these characteristics. The Committee is conducting a ring-trial to finalize FDA qualification for this application. This presentation will summarize lessons learned from these experiences, which we hope facilitates regulatory adoption of future biomarkers.

Presentation: Oral

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Japan's approach for applying MPS as a wet-simulator in chemical risk assessment

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Chemical risk assessment, for which alternative methods to animal tests are being developed, is now introducing IATA, which combines several *in vitro* tests developed based on AOP key events. Consideration of weight of each test for their combination, it is important to predict the kinetics and the distribution of chemicals in the body and tissues. Currently, applications of prediction systems based on *in silico* models are being proposed, but most of them have been developed by clinical data of pharmaceutics. As human pharmacokinetic data of chemical substances are scarce, the development of wet-simulators is helpful to obtain parameters for *in silico* model prediction. MPS is one of such candidates. In Japan, the AMED-MPS2 project, led by AMED (Japan Agency for Medical Research and Development), is developing a first-pass model utilizing gut-liver co-culture MPS developed by Japanese research laboratories. The "points to consider" for the implementation of this model to industry and regulation sections, which are discussing in another AMED project: MPS-RS (regulatory science) project, will be presented along with their evaluation methods and results of the study.

References

[1] Ishida (2021). Research and development of microphysiological systems in Japan supported by the AMED-MPS project. *Front Toxicol*.

[2] Horiuchi, Kuroda, Komizu et al. (2023). Consideration of commercially available hepatocytes as cell sources for liver-microphysiological systems by comparing liver characteristics. *Pharmaceutics*.

Presentation: Oral

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Spinosad: A physiologically-based pharmacokinetic (PBPK) model in the rat and human including the pregnancy life-stage

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A physiologically-based pharmacokinetic (PBPK) model was developed in R for the insecticide Spinosad (mixture of Spinosyns A and D). The model simulates dosimetry for rats and humans, including physiological changes during pregnancy. Treatment with Spinosad was associated with parturition (dystocia) in a 2-generation rat study at 100 (but not at 3 or 10) mg/kg bw/day; further mechanistic work demonstrated these are potentially receptor-mediated (threshold) effects on rat uterine contractility. The goal of this PBPK model was to estimate uterine concentrations during pregnancy for rats and humans. Standard parameters and equations were used for organ volumes, cardiac output, and tissue blood flow, including how these change during pregnancy. The model simulates saturable metabolism of Spinosyn A and Spinosyn B (the two most prevalent "spinosyn" factors observed *in vivo*), including the formation of Spinosyn B from A via saturable elimination of CYP-based metabolism as well as biliary clearance (primarily as direct glutathione conjugation). The model was fit using blood, plasma, and uterine concentration data from a single dose rat gavage study at gestation day 20 (GD20) and validated using a repeat dose rat gavage study from GD17-GD20, a rat dietary study from GD6-GD20, and a dermal exposure study among healthy adults. The sum of the Spinosyn A and B mean uterine concentrations for rats was approximately 5- to 15-fold higher than the predicted values for humans, suggesting that the uterine concentrations are higher for rats compared to humans in a dose level range corresponding to the rat 2-generation NO(A)EL and LOAEL.

Presentation: Oral

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Critical role of toxicokinetics and ADME methods in quantifying exposure for next generation risk assessment

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Besides ethical considerations, scientific reasons have led to a worldwide shift towards human health safety assessments of chemicals without using animal testing. The Next Generation Risk Assessment (NGRA) approach is exposure-led and hypothesis-driven, based on the combination of non-animal data derived from *in silico*, *in chemico* and *in vitro* methodologies named "New Approach Methodologies" or "NAMs". To come to a reliable assessment within NGRA, an estimate of the internal exposure should be provided. That requires implementations of a variety of ADME tools (*in vitro* parameterization and *in silico* simulations) to convert human external exposure from multiple routes (dermal, inhalation) to concentration-time profiles within blood and specific organs. In this presentation, the progress towards modern ADME tools will be shown and it will be demonstrated how its application impacts highly on successful NGRA of chemicals, e.g., UV filters. Additionally, the roles of ADME, *in vitro* and *in silico* simulations, on internal Threshold of Toxicological Concern (iTTC) will be discussed. Furthermore, the possible impact of Lab-on Chip on toxicokinetics and its possible benefit on NGRA will be briefly discussed.

Presentation: Oral

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Leveraging big data and distribution approaches for environmental risk assessment

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Risk assessment of chemicals involves an assessment of toxicity, exposure and the resulting likelihood of observing an adverse response. It also requires ethical and resource consideration as to how much data are attainable and should be derived (e.g., via use of an-

imal testing) versus what is considered an acceptable level of extrapolation. The threshold for toxicological concern (TTC) can be used to establish an exposure level below which no risk is expected based on existing data for chemicals within a similar grouping. TTCs have been used in various human health applications for many years, however, application in the ecological space is relatively new. The EnviroTox database was built specifically to explore the application of this concept, bringing together over 80K aquatic ecotoxicity data for 4200+ chemicals across 1641 aquatic species. This tool also allows for the building of statistical distributions of either toxicity hazard values (chemical toxicity distributions; CTD) or PNECs (ecological TTC; ecoTTC). This presentation will highlight the use and application of these approaches with a case study on water quality criteria for benzene-type narcotic chemicals. HC5 values for each distribution method were derived for each group were derived using the EnviroTox database and compared to corresponding regulatory values. This analysis allows a quantitative evaluation of the degree of conservatism in ecoTTC and CTD approaches and explores the potential for grouped methods to derive criteria values.

Presentation: Oral

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Keeping toxicology education relevant in the 21st century

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In vitro and *in silico* expertise are increasingly necessary in the field of toxicology; however, outdated graduate curriculums are not providing students with the skills needed to excel in today's landscape. This has led to a need to re-examine current education practices with the intention to update undergraduate and graduate education programs such that early career scientists enter the work force with fundamental training in modern, scientifically relevant test methods, as well as a basic understanding of regulatory implementation of new methods for decision-making. This presentation offers insight into opportunities to 1) provide educators with the help needed to update their toxicology program courses and curriculums, 2) bring awareness to existing forums to connect educators with toxicologists who have expertise in *in vitro* and *in silico* testing, and 3) improve outreach between students and researchers who leverage cutting-edge technologies that improve public health and environmental safety. The presentation will be of interest to students and established scientists from any sector interested in sharing modern approaches to safety assessment with students, so that together we can build a roadmap to train the next generation of toxicologists.

Presentation: Oral

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The current and future role of OECD in chemicals assessment

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The OECD has been an international hub for the harmonization of toxicology methods for evaluating the potential hazard of chemicals to humans and the environment for 50 years. Currently OECD devotes considerable resources to promote and eventually standardize new approach methods (NAMs), and to develop guidance for conducting studies, reporting and using NAMs in regulatory decision-making. I will reflect on recent advances of the OECD Adverse Outcome Pathway (AOP) Programme and how it helps developing frameworks for organizing complex data and designing integrated approaches to testing and assessment. With member countries, OECD is also developing an ecosystem of interoperable, freely accessible electronic tools that facilitate sharing of data and predicting chemical effects. OECD has also recently published a number of standardized reporting templates to increase the uptake of a variety of NAM data. Ultimately, I will describe the roadmap to streamline the review, validation and standardization of new approach methods for use in regulatory decision-making and discuss the role of OECD in the envisioned future of chemicals assessment.

Presentation: Oral

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A complete curriculum: Introducing the 3Rs in secondary school

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To improve animal welfare and reduce reliance on animal models in science and education, an effort must be made to teach the next generation about the 3Rs principles – replacement, reduction, and refinement of animal use – and progress that is being made with non-animal models in scientific research and education. We need a structured plan that introduces students to humane research methods before they enter university, where reliance on animal models is normalized.

To address this need, we assembled a team of teachers, subject matter experts, and curriculum developers to design a humane education curriculum: "Animal Use in Science: Exploring the 3Rs." The curriculum introduces secondary school students to the 3Rs

and covers how animals are used in science, testing, and education in the United States.

The curriculum consists of eight modules that explore the 3Rs and related topics such as the use of animals in cosmetics testing, the environmental impact of using animal models, and legal and regulatory guidance for the use of animals in research. The modules can be taught separately or in combination, giving educators flexibility to choose their focus. All of the modules were designed to align with key national standards in the U.S., including Next Generation Science Standards.

During the session, we will share curriculum teaching plans, learning materials, and performance assessment tasks with attendees. In addition to showcasing traditional handouts and slide decks, we will also share interactive eLearning modules developed to augment the curriculum.

Presentation: Oral

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New Korean legal mandates on IACUC qualification and compliance training programs to enhance the Three Rs principles: 2022 amendment of the Animal Protection Act

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Over the past several decades, good research practice with laboratory animal welfare has become a societal and political concern in the field of science nationally and globally. However, governing rules for the use of laboratory animals are contingent on the research culture and traditions. While the Animal Protection Act in Korea was enacted in 1991, a framework for oversight of laboratory animal care and humane use applying the Three Rs and ethical review in the research, testing, and education has been applied since 2008. Based on the 2021 government annual report, 481 IACUCs were registered, and 4,880,252 animals were used in Korea. Driven by both scientific needs and public concerns relating to responsible science and minimizing animal harm, a total amendment of the Animal Protection Act will be enforced from 27 April 2023. To be a legally qualified IACUC member, one must show a 4-hour compliance training certificate conducted by the Animal and Plant Quarantine Agency or a designated education provider. In one of the Animal Protection Act amendments, additional training requirements for the acting IACUC members are enforced. The BIC Study Foundation, a certified IACUC member compliance training provider, has issued over 1,500 certificates since 2018 and reported these records to the Animal and Plant Quarantine Agency quarterly. This paper will summarize the key changes of the Animal Protection Act relating to animal use and lessons learned from our IACUC member training program aiming to enhance regulatory compliance with the Three Rs principles.

Presentation: Oral

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Vascularization of multi-organ-on-chips with blood and lymphatic endothelial cells for the generation of immunocompetent skin models

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Background: 3D human skin models fail to include both blood (BEC) and lymphatic (LEC) endothelial cells despite their essential role for homeostasis and immune responses, limiting their relevance for disease modeling and safety testing.

Aim: Establish a method for vascularization of organ-on-chip microfluidics with human BECs or LECs which allows long term culturing under physiologic flow conditions for immune competent (multi-) organ-on-chip models.

Methods: Primary human skin endothelial cells were separated into BECS and LECs, expanded to generate, used to vascularize multi-organ-on-chip microfluidic bioreactors and cultured for 14 days under dynamic flow mimicking blood and lymph flow pressures.

Results: Large numbers of highly pure BECS and LECs were obtained and used to vascularize organ-on-chip devices. Upon prolonged culture, cells retained their endothelial specific phenotype. Mimicking blood vessel flow induced morphological changes as cells aligned in the direction of the flow, while this does not occur when applying lower lymphatic vessel flow. BEC and LEC biomarker expression of was clearly different but not influenced by flow conditions. BECs and LECs respond to inflammatory conditions by upregulating soluble ICAM, VCAM, CCL2 and IL-6. While mRNA levels of endothelial junction markers (Cldn5, VEcadh, ZO1) did not change in BECs and LECs, the LEC specific marker Prox-1 was clearly reduced in LECs upon inflammatory conditions.

Conclusion: The presented method can be used to further enhance organ-on-chip models through the incorporation of func-

tional blood and lymphatic endothelial cells. This will result in relevant healthy and diseased tissue models to investigate human disease and safety testing.

Presentation: Oral

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The right tool for the job: Why and how to adapt your science communication for in-person and virtual events

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More than ever, the challenges science communicators face nowadays remain daunting: they need to reach out to various audiences with different interests, simplify the message without losing accuracy and engage multiple communities.

To overcome these hurdles, applying different tools is crucial since each is best suited for specific situations. Moreover, in-person events could work better than virtual ones depending on the communication objectives, even if they add new challenges.

Unfortunately, there is no secret recipe to master every action within science communication. Much trial and error are often required to learn which is best for each situation.

In this talk, I will describe the formats encountered and practiced in my journey in science communication, as well as recent experiences with two innovative tools for science communication in toxicology and animal replacement: the live streaming show TOXstreams and the card game TATAbox.

I will focus on the differences between in-person and virtual events, drawing from my experience. For example, my activities with the science outreach organization Pint of Science Belgium and my participation in the "I love science festival" in Brussels during my professional work.

At the same time, I will describe my experience with podcasts and videos. More specifically, my live streaming show TOXstreams merges elements from live talk shows, journalistic emissions and stand-up comedy.

Science communicators need to understand that each person we engage with is different, and only practicing with new activities will help us and our community to grow and keep the momentum.

Presentation: Oral

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Development of an internal granting program for proactive 3Rs advancement

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Novartis scientists expressed a desire for a program which provided resources to encourage 3Rs initiatives that would also benefit their research, increase awareness of the 3Rs, and improve data quality. Novartis leadership supported this request by establishing a program supporting the proactive development or incorporation of 3Rs techniques or technology into the drug discovery and development research process: the Innovation in 3Rs Grant program. In its inaugural year, the program received proposals from 4 global sites and 16 disease or functional areas, spanning each of the 3Rs. Applications were first evaluated on creativity and impact by an internal scientific review board. Selected applications next met with research stakeholders, such as the IACUC and biostatisticians, where applicable. Finalists then presented a detailed plan of their project and were evaluated for scientific rigor and feasibility. Five projects were ultimately selected for funding. Three applications utilized non-animal models (organoids, organ on a chip, and AI software) that will Reduce the number of animals needed for that type of research. Two projects investigated new strategies to improve post-operative care of mice and identify earlier humane endpoints to Refine and improve the animal's experience on study. Grant recipients and their scientific line managers report the grants de-risked piloting new technologies and methodologies that may not only bring 3Rs advancements to their research but also improve operational efficiency and enhance data quality. The Innovation in 3Rs Grant program is a unique approach to inspire and support scientists to explore new 3Rs approaches in drug discovery.

Presentation: Oral

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Professional advantages of AOP development for graduate students

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In regional universities, graduate program education needs to be offered to part-time students. For example, 56% graduate students (2,063 out of 3,715) are part-time students at Rowan University in 2023. These graduate students have full time jobs and can only use

free periods to take courses and even work for research projects. The urgent requirements of these students are not only a flexible course schedule in the evenings but the feasibility to finish the research works off campus.

In the past decade, the adverse outcome pathway (AOP) framework has provided mechanistic extrapolations of chemical toxicity. The AOP is a theoretical concept linking molecular initiating events to adverse outcomes through intermediate key events. There are many AOP data sources, modeling tools and education materials developed, and publicly available. There have been over 100 graduate students enrolled in my informatics class in the past eight years. The current available AOP resources, such as PubChem, AOP-Wiki, ToxCast and etc., were extensively involved in this class. The chemistry, biology, toxicology and computational components of AOP are well suitable for multidisciplinary students. Although the students are required to attend in lectures on campus, they are able to finish assignments at home using the online resources. Furthermore, ten students chose the computational toxicology area to get their master or PhD degrees. These efforts resulted in 15 peer reviewed scientific research papers of AOP modeling. The AOP research, which can even be performed at home, greatly advanced and strengthened the newly graduate programs in Rowan University.

Presentation: Oral

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Development of a microphysiological skin-liver-thyroid Chip3 and its application to evaluate the effects on thyroid hormones of topically applied cosmetic ingredients under consumer-relevant conditions

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All cosmetic ingredients registered in Europe must be evaluated for their safety using non-animal methods. Microphysiological systems (MPS) offer a more complex higher tier model to evaluate chemicals. We investigated whether thyroid follicles could be incorporated to evaluate the potential of topically applied chemicals to cause endocrine disruption. This combination of models in the HUMIMIC Chip3 is new; therefore, we describe here how it was optimized using two chemicals known to inhibit thyroid production, daidzein and genistein. The MPS was comprised of Phenion® Full Thickness skin, liver spheroids and thyroid follicles cocultured in the TissUse HUMIMIC Chip3. Endocrine disruption effects were determined according to changes in thyroid hormones, thyroxine (T4) and 3,3',5-triiodothyronine (T3). The skin-liver-thyroid Chip3 model was used to determine a consumer-relevant exposure to daidzein present in a body lotion based on thyroid effects. A "safe dose" of 0.235 µg/cm^2, i.e., 0.047% applied in 0.5 mg/cm^2 of body lotion was the highest concentration of daidzein which does not result in changes in T3 and T4 levels. This concentration correlated well with the value considered safe by regulators. In conclusion, the Chip3 model enabled the incorporation of the relevant exposure route, metabolism in the skin and liver, and the bioactivity endpoint into a single model. These conditions are closer to those *in vivo* than 2D cell/tissue assays lacking metabolic function. Importantly, it also allowed the assessment of repeated doses of chemical and a direct comparison of systemic and tissue concentrations with toxicodynamic effects over time, which is more realistic and relevant for safety assessment.

Presentation: Oral

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Evaluating skin sensitization hazard of diverse chemicals using GARDskin

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Multiple U.S. agencies require that chemicals be assessed for skin sensitization potential, although specific requirements vary based on the remit of an agency's chemical evaluation and management programs. Determination of skin sensitization hazard and potency using data from non-animal models adopted by OECD is gaining interest. The recently accepted GARDskin is the first assay based on genomics and machine-learning algorithms to make hazard predictions. Validation of the GARDskin required openness and transparency to verify the performance of the prediction model. NICEATM and collaborators tested 30 "challenging" chemicals in the GARDskin assay to potentially expand the applicability do-

main of the assay. Chemicals were nominated by NTP, EPA, the U.S. Consumer Product Safety Commission, and FDA. Results were compared to historical data from the mouse local lymph node assay (LLNA). Concordance of hazard classifications based on the LLNA was determined for classifications based on outcomes from three *in vitro* tests: the human cell line activation test (h-CLAT), direct peptide reactivity assay (DPRA), and KeratinoSens assay (KS). Against the LLNA, the GARDskin had sensitivity of 86%, specificity of 42%, and accuracy of 64%. Comparatively, the DPRA, h-CLAT, and KS performance metrics ranged from 40-54% for sensitivity, 22-47% for specificity, and 31-50% accuracy. Overall, the GARDskin predicted LLNA-based classifications with higher concordance than the other *in vitro* assays and appears to be useful for predicting skin sensitization hazard. Assessment of the suitability of GARDskin to be included in OECD Test Guideline 497 is ongoing.

Project funding provided under NIEHS Contract Nos. HHSN273201500010C and HHSN27320140017C.

Presentation: Oral

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Development and curation of an acute inhalation toxicity database

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Interest is increasing in using computational modeling tools to predict toxicity endpoints for regulatory decision-making. Developing such tools requires robust, well-curated, and chemically diverse training data. NICEATM has compiled and curated rat acute inhalation data for approximately 1700 chemicals from a variety of sources, including ECHA, EPA, the U.S. National Institute for Occupational Safety and Health, the U.S. Department of Defense, and PubChem/ChemIDPlus. We used manual and automated curation techniques to extract LC50 values and/or ranges. Metadata collected for each entry included exposure type, exposure route, species, sex, and number of animals tested. Duplicate studies were removed. All qualifying LC50 values were converted to 4-hour exposures using Haber's Law to allow for direct comparison to, and assignment of, GHS categories. For many studies, details were not available on whether the substance was delivered via aerosol, vapor, or gas. Therefore, a rule-based decision process was applied to determine the GHS category of each chemical based on its physicochemical properties. The data were analyzed for variability across categories for chemicals with multiple studies and exposure types. The curated data will be made publicly available and used for a collaborative modeling effort to predict continuous, binary, and multicategory endpoints based on the regulatory use of the LC50 value for hazard assessment. The development and curation of this acute inhalation database for consensus models will support progress in the use of NAMs for regulatory decision-making.

Project was funded by NIEHS under Contract No. HHSN273201500010C.

Presentation: Oral

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Defined approaches for GHS categorization to assess eye irritation potential of agrochemical formulations

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Accuracy of non-animal alternatives for assessing eye irritation has historically been determined by comparison to the Draize rabbit eye test. However, because of the rabbit test's demonstrated lack of reproducibility and human relevance, there has been movement away from evaluating alternatives via one-to-one comparisons with it, in favor of evaluating based on the reliability and human-relevance of the method. We used a common set of non-animal assays in a multi-phase study to assess the eye irritation potential of agrochemical formulations. Test articles represented major agrochemical formulation types and the complete range of GHS eye irritation hazard classifications. Assays were selected for inclusion based on their relevance to humans, and results were assessed to determine which assays should advance to subsequent testing phases. A total of 29 formulations were tested in as many as five assays: bovine corneal opacity and permeability (with histopathology), EpiOcular, SkinEthic Time-to-Toxicity, *in vitro* depth of injury, and EyeIRR-IS. Data generated were used to analyze alignment of predictions across non-animal assays and the rabbit test. Consensus GHS predictions were determined based on majority alignment among individual assay results (achieved for 27 formulations). Interestingly, the historical rabbit test classification differed from the consensus prediction for five formulations. This suggests that the rabbit test may not be a suitable reference method for deriving GHS eye irritation hazard classifications for

agrochemical products. These data will support ongoing work to develop defined approaches for assessing eye irritation potential of agrochemical formulations.

Project was funded by NIEHS under Contract No. HHSN273201500010C.

Presentation: Oral

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Norecopa: A one-stop-shop for global 3R resources

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Today's scientists face a constant "Niagara Falls" of information about novel methods which may advance the 3Rs – via scientific papers, congresses and social media, to mention a few. Norecopa offers an up-to-date, global overview of quality resources, organized in one large database with a sophisticated search engine (https://norecopa.no). The 9,000+ page website includes many data sets, some from the EU Commission and others produced by Norecopa itself. Among these are 3R Guide (an overview of over 400 guidelines for animal research), NORINA (alternatives to animal use in education and training, at all levels from schools to university and for research animal users) and EU collections of NAMs within cardiovascular and respiratory research. English-language newsletters are issued 7-8 times a year to inform users of the latest developments.

The site also hosts the PREPARE guidelines for planning research: a checklist (in 34 languages) and a website with links to resources for each item.

Norecopa also maintains a Refinement Wiki for those who want a portal for rapid dissemination and discussion (https://wiki.norecopa.no), the website of the International Culture of Care Network (https://norecopa.no/coc), and an International Webinars and Meetings Calendar (https://norecopa.no/calendar).

Norecopa regularly holds presentations about these resources and has also produced a downloadable 40-page slide deck about the 3R principle.

Norecopa is an independent member organization, with a Board representing all four major stakeholders in animal research: regulatory authorities, industry, research and animal welfare. Norecopa aims to advance the 3Rs through consensus between these stakeholders, promoting understanding for the 3R principle.

Presentation: Oral

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Marseille Declaration: Together we prioritize animal welfare

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It is fundamental that high standards of animal welfare and quality of work are maintained in the use of animals in science, without exception, for two reasons. First, because there is a moral obligation to avoid animal suffering. And second, because only psychologically and physiologically healthy animals provide meaningful and reliable data. This health can only be achieved if husbandry conditions ensure that the basic species-specific needs for a complex environment that provides adequate space for movement, social contact, nutrition, stimulation, and freedom from stress and disease are met.

Local legislation varies widely and is insufficient to ensure that these requirements for appropriate animal husbandry are met worldwide. For this reason, and to enable coordinated action with our partners in industry and academia, the signatories of the Marseille Declaration have agreed to set out their objectives and priorities about the welfare and husbandry conditions of laboratory animals.

The declaration is named because the initial signatories – Merck, Novartis, Novo Nordisk and Sanofi – agreed to this framework at the 2022 FELASA conference in Marseille. The declaration does not claim to be a concrete guideline or audit standard, nor does it claim to be complete. Rather, in 11 short sections, it outlines basic requirements that sometimes go beyond local legislation and that the signatory companies have defined as their expectations of external partners working with animals on their behalf worldwide, including the requirement to meet at least the standards set by the EU for NHP, dogs and pigs.

Reference

https://www.merckgroup.com/en/sustainability/business-ethics/marseille-declaration.html

Presentation: Oral

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Working towards the "essential 10"

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Within the laboratory animal sciences, we have some great best practice guidelines for planning (the PREPARE guidelines) and reporting (ARRIVE 2.0) animal studies. The ability to report "the essential 10" as defined within ARRIVE 2.0 (1. Study design; 2. Sample size; 3. Inclusion and exclusion criteria; 4. Randomisation; 5. Blinding; 6. Outcome measures; 7. Statistical methods; 8. Experimental animals; 9. Experimental procedures; 10. Results) does not happen by chance. It requires planning, excellent communication and input from all members of the scientific and animal care team. In this presentation I will discuss what delivery of the "essential 10" means in practice. I will share freely available tools and resources to support implementation of the "essential 10", plus some hints and tips that I have picked up during my own career working within the laboratory animal science sector.

Presentation: Oral

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Europe enforces and supports *in vitro* methods

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Pharmacopeias require a safety test for the exclusion of pyrogens (fever-inducing substances) in drugs, as these may induce life-threatening consequences in patients. Testing was initially performed by the Rabbit Pyrogen test (RPT, animal test), and later largely replaced by the Bacterial Endotoxin Test (BET). The BET is not an animal test, but still its reagents are derived from endangered wild-life species of horseshoe crabs.

Since 2010 the European Pharmacopeia (PhEur) includes the General Chapter 2.6.30. Monocyte Activation Test (MAT) as true *in-vitro* replacement of the RPT. Additionally in 2021 a recombinant version of BET (rFC) was introduced as General Chapter 2.6.32.

The lack of MAT-implementation (which contradicts EU-Directive 2010/63) prompted the PhEur and its expert groups in 2021 to start a whole new strategy for Pyrogen testing. The RPT will be deleted from 60 texts, and reference to the new Chapter 5.1.13. Pyrogenicity will be made. Until 2026 the RPT will vanish from the PhEur. In 5.1.13. the MAT is the new test for pyrogens in Europe, for Endotoxin testing both classical BET or the new rFC can be used.

By this remarkable exercise both the MAT and the rFC become full compendial in Europe, and MAT is the new standard for pyrogen testing. MAT and rFC are the new reality, and the future of global Pyrogen and Endotoxin testing. Other Pharmacopeias will follow sooner or later. The FDA modernization act and the dramatic changes at USP give us hope.

Presentation: Oral

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Educating risk assessors in the probabilistic risk assessment approach

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Despite our growing appreciation of the complexity of both the dose-response relationship as more granular data for exposure parameters, human health risk assessment for most regulatory purposes is still done in a largely deterministic way, with a heavy reliance on averages for environmental concentrations, body weight, and estimated intake to produce a simple point estimate for risk. Any uncertainty within the data is simply dealt with by adding uncertainty factors. Probabilistic risk assessment seeks to change this by dealing with uncertainty and variability more explicitly, producing not a point estimate but a range outcomes and probabilities. However, this will almost certainly require new skill sets for risk assessors. For one, probabilistic risk assessors need to be equipped with statistical literacy to handle more complicated data. Additionally, as toxicology switches to a more mechanistic, qAOP focus, this will require a more solid grounding in quantitative biology – a trend that is true for all the life sciences. Most importantly, probabilistic risk assessment approaches require literacy in machine learning and data-driven approaches. Not every toxicologist or risk assessor needs to be an expert in deep learning or Bayesian statistics – in fact most do not – but they will require understanding of how AI uses data and how to make sense of the outputs beyond simple summary statistics, how to benchmark AI performance, and how to understand both the strengths and weaknesses of AI approaches.

Presentation: Oral

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Strengthening the weight of evidence for fish embryo toxicity (FET) data to replace acute fish toxicity (SWiFT)

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The acute fish toxicity test (AFT; OECD TG 203) is currently required for chemical hazard and risk assessment purposes according to many different chemical legislations and regulations. The fish embryo toxicity test (FET; OECD TG 236) has been proposed as an alternative to using juvenile fish to reduce the number of live animals required for hazard and risk assessments of chemicals. However, FET data are not yet accepted as a replacement for AFT data for regulatory purposes such as REACH. The European Chemicals Agency has recommended the development of a Weight of Evidence (WoE) approach for strengthening the ability of FET data to predict (juvenile) acute fish toxicity. To meet this challenge, we have developed a Bayesian network model (SWiFT) for using FET data with multiple lines of evidence in a probabilistic WoE approach. A Bayesian network is a probabilistic graphical model that represents a set of variables and their conditional dependencies via a directed acyclic graph. The additional lines of evidence in this Bayesian network include data from fish gill cell-line cytotoxicity assay, neurotoxicity, biotransformation, data on other organisms, read across and *in silico* predictions. As additional evidence is included in the SWiFT model for the prediction of a given chemical's ecotoxicity profile, both the accuracy and the precision of the predicted acute fish toxicity increases. The SWiFT model not only accurately predicts acute fish toxicity, but it also provides a tool for improving environmental hazard assessment of chemicals.

Presentation: Oral

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Fun with NAMs

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Education brings neither glory to the scientists nor a better h-index. Nevertheless, it is useful for multiple reasons such as knowledge sharing, capacity building and creation of an adequate ecosystem. Overall, one can admit that the education and training about 3Rs at university level has the merit to exist even if it could be possibly better advertised and communicated. The JRC launched a mapping exercise on this matter in 2018 but as far as the authors are concerned the results of the study were not published [1]. A category of individuals that is rarely targeted properly is the general public as well as teaching at primary and secondary school. JRC took care of the latter by providing learning scenarios to empower the teachers [2]. Moreover, organising open days as well as participating in science festivals are great venues for reaching out the general public. Still, there is space for creativity by providing other formats. At Altertox, a new concept and format is expected to complement the current "arsenal" of tools available. TATAbox is the first serious game meant to open a conversation about NAMs (New Approach Methodologies) and validation process in a fun and convivial environment. "TATABOX" (Towards Alternatives To Animal box) tiles are not meant to be exhaustive in terms of content as well as persona but rather a starting point for discussion with concrete items within a team on the process towards regulatory acceptance.

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[2] Introducing the Three Rs into secondary schools, universities. https://publications.jrc.ec.europa.eu › JRC123343

Presentation: Oral

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EcoToxChip test system: A toxicogenomic new approach method (NAM) for chemical prioritization and environmental management

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Since 2016 our team has set forth to develop, test, validate, and commercialize toxicogenomic solutions (i.e., EcoToxChips, and a data evaluation tool EcoToxXplorer.ca) for the characterization, prioritization, and management of environmental chemicals and complex mixtures of regulatory concern. EcoToxChips have been developed for early life stage, ELS (and *in vitro*, when possible) model species representing key vertebrate groups in ecological risk assessment (fish-fathead minnow and rainbow trout; bird-Japanese quail and double-crested cormorant; amphibian-*Xenopus laevis* and Northern leopard frog). To date, more than 1,800 EcoToxChips have been analyzed, and in this presentation we will

summarize key findings from our case studies covering 28 distinct chemicals and 4 complex environmental samples with a focus on: a) technical reproducibility; b) identification of chemical mechanism of action; c) derivation of transcriptomic points of departure. Taken together, these diverse studies demonstrate how the EcoToxChip Test System can serve as a toxicogenomic New Approach Method that is accessible, standardized and user-validated. Importantly, many partners have regulatory interests, and so the case studies are helping us to purposefully design the Test System to be one that can be used quantitatively as a decision support tool in regulatory applications including chemical prioritization and guideline development.

This study was conducted as a part of a large-scale Genome Canada-funded project (EcoToxChip project – www.ecotoxchip.ca).

Presentation: Oral

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KoCVAM's accomplishments in development of the alternative methods listed in the OECD TGs and support for the introduction and utilization of non-animal alternative methods in Korea

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Korean center for the Validation of Alternative Methods (KoCVAM) has been operating within the Ministry of Food and Drug Safety (MFDS) since 2009. KoCVAM has exchanged opinions and information with the ICATM partners in development and validation of alternative methods and provided inputs to draft OECD TGs. There are several acts in Korea (i.e., Cosmetics Act, etc.) that encourage the use of alternative methods for chemical testing and assessment. To promote alternative methods and support the utilization of the methods in Korea, KoCVAM has published and provided education on guidelines based on the OECD TGs. In addition, KoCVAM has held annual training workshops on alternative methods to help domestic research institutions including GLP facilities to utilize the methods. In addition, KoCVAM has contributed to enhancing the competitiveness of the domestic industry by helping list the alternative test methods developed and validated in Korea in the OECD TGs (TG442B, TG492 and TG439). KoCVAM will continue to spearhead the promotion of alternative methods based on the 3R Principles.

Funding Source: This research was supported by a grant (23214MFDS262) from Ministry of Food and Drug Safety in 2023.

Presentation: Oral

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The ecological risk classification approach for prioritizing organic substances in Canada: One giant leap for NAM-kind

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ECCC has recently developed version 2.0 of the ERC (ERC2) for re-evaluating ~12,200 organics on the Canadian Domestic Substances List (DSL), which were not "categorized" as a concern using PBT metrics in 2006. ERC2 continues as a risk-based approach for prioritizing substances for future chemicals management activities such as risk assessment. The approach integrates many types of NAMs inside a confidence-based framework using rule-based logic routines to find consensus within and among *in silico*, *in chemico*, *in vitro* and *in vivo* data to classify hazard, exposure and risk as well as assign confidence and severity (a scaling metric) scores. All ERC2 data are contained in chemical profiles searchable by CAS Registry Number (CAS RN). ERC2 is capable of profiling both a target chemical and its metabolites for certain hazard endpoints. Hazard data are organized according to the adverse outcome pathway (AOP) framework to provide plausible mechanistic reasoning for explaining adverse outcomes thereby increasing regulatory confidence. Cross-species susceptibility is taken into account when examining mechanistic data. Exposure data are organized at different spatial and temporal scales and used to help understand short vs long-term exposure using a regional scale multimedia model. This presentation will pinpoint NAM integration in core ERC2 functionality. Results for risk assessment priority setting, using a confidence-severity matrix and performance vs other hazard and risk approaches, is also presented.

Presentation: Oral

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Restricted space does not have to mean restricted choice – Keeping monkeys happy!

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The use of nonhuman primate models is pivotal to investigate drugs and/or vaccines for highly infectious pathogens prior to use in humans, especially when naturally occurring outbreaks of disease are rare and unpredictable. The most infectious and deadly pathogens need to be handled in high containment laboratories (Biosafety level 3 and 4) and therefore strategies are required to safely house infected animals whilst providing the animals with psychological support, mental and physical stimulation and promoting natural behaviours. Callithrix jacchus (common marmosets) are the nonhuman primate of choice at Dstl. Marmosets are housed in mixed-sex pairs in bespoke cages with a variety of environmental enrichment. When animals are infected with pathogens, these cages are contained within negative-pressure, half-suit isolators. Studies are refined by employing humane endpoints to reduce suffering which is supported by the use of remote telemetry and CCTV. Positive reinforcement is used to train/condition animals to accept placebo in a milkshake using a syringe for oral administration studies. Similarly, they are conditioned to voluntarily sit on a bucket for weighing purposes. A novel, reversible anaesthesia regimen has also been established to reduce the impact associated with repeated procedures. Despite the restrictions associated with working in containment, a carefully considered approach can provide animals with appropriate stimulation whilst enabling the delivery of high-quality science.

Presentation: Oral

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Accepting the separation of toxicology and statistics in new approach methodologies in ecological risk assessment

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As we move away from animal testing, we move towards a future of models. In ecotoxicology, this requires a shift in the roles of toxicology and statistics; one that is underway yet faces resistance by many who find themselves uncertain on how to view the defensibility of models poised to replace familiar animal tests. The leading edge of toxicology has shifted towards cellular and molecular endpoints, which require models to translate to biological responses applicable for risk assessors. Statistical models based on probability and data trends become more important in the analysis phases of ERA to help quantify uncertainty around numeric threshold values. In a reality where no model is "right", how do we agree when models are useful for us to succeed in reducing animal testing and protecting the environment? Using Interspecies Correlation Estimation (ICE) models for extrapolation of acute toxicity in aquatic organisms as an example, the pros of simplicity and quantifiable accuracy are weighed against the unknown, unquantifiable uncertainties of lost toxicological mechanisms. The validity of ICE models has been demonstrated by multiple, independent researchers, yet toxicologists struggle with the absence of toxicological mechanisms and model acceptance, creating impasse on moving the state of the science forward. The value and uncertainties of ICE models are weighed against the environmental consequences associated with limited data and inaction of government agencies. The challenges reviewed here highlight larger future challenges: As the ecotoxicology community remain at odds over simple statistical models, how will more complex NAMs ever be accepted?

Presentation: Oral

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Review of animal testing requirements in WHO guidelines and recommendations for biologicals: A proposal to implement 3Rs principles

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Biologicals such as vaccines, cytokines, enzymes, and hormones are tested extensively post-licensure as part of routine quality control and batch release testing with an estimated 8 million animals a year used worldwide for this purpose. This animal use puts a significant financial burden on manufacturers and national control laboratories, is time and resource intensive, and the methods themselves can cause significant pain and distress to the animals.

The World Health Organization (WHO) is mandated to establish international standards for this purpose and their guidelines and recommendations carry significant global influence. However, a review of the animal testing requirements within these guidelines has never been conducted and there may be opportunities to adopt the latest non-animal technologies that are being missed as a result.

In 2019, the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) were tasked by the WHO and funded by the Bill & Melinda Gates Foundation to carry out an independent and comprehensive review of WHO guidelines for biologics to determine which animal tests are currently recommended for the batch release testing of biologics and vaccines and to identify opportunities for better implementation of 3Rs principles and alternative test methods. A comprehensive report was delivered to the WHO in June 2023 and will be formally presented to WHO Expert Committee on Biological Standardisation in October 2023.

This presentation will highlight the key findings and recommendations from the review and signpost key resources that NC3Rs have created to support this project.

Presentation: Oral

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Curating chemical use and exposure predictions to contextualize chemical hazard

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To translate chemical hazard predictions into risk, it is essential to understand how populations interact with and are exposed to various chemical sources. High-throughput *in silico* exposure simulations and chemical use models can inform exposure scenarios for data-poor chemicals, but the large data volumes associated with these tasks can be challenging for those unfamiliar with computational methods. To provide easily interpretable and accessible exposure and use data, we integrated exposure predictions from EPA's SEEM3 models and use categories from EPA's Chemical and Product Database (CPDat) into the Integrated Chemical Environment v4.0 (ICE; https://ice.ntp.niehs.nih.gov/). Population-level estimates for the 5th, 50th, and 95th percentiles of exposure were obtained from SEEM3, and pathway-specific outputs were used to generate near- and far-field annotations. Predictions were restricted to chemicals within the SEEM3 models' applicability domain. Chemical categories in the ICE Chemical Characterization tool were expanded to include updated consumer use data and functional use from CPDat. Reported functional use terms for nearly 9,500 chemicals were harmonized to OECD functional use categories based on suggested synonyms and expert opinion. To characterize potential use for over 100,000 chemicals that lacked reported functional use, we also added predicted functional use from CPDat to ICE. These predicted uses were curated using a high-probability cutoff to ensure high confidence in results. When presented alongside other toxicologically relevant data within ICE, the addition of these highly curated data provides users with a more comprehensive context for chemical hazard.

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Presentation: Oral

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Update on recent activities at JaCVAM

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The Japanese Center for the Validation of Alternative Methods (JaCVAM) was established to fulfill the CBSR's responsibility to protect the general public by assessing the safety of chemicals and other materials as specified in NIHS regulations, while replacing, reducing, or refining (the 3Rs) the use of animals wherever possible by promoting the use of alternative methods to animal testing in regulatory studies. Since 2005, JaCVAM has contributed to establish more than ten Test Guidelines (TGs) in Organisation for Economic Co-operation and Development (OECD) supported by the International Cooperation on Alternative Test Methods (ICATM) members. Recently, JaCVAM is developing test methods on broadly defined immunotoxicity. We are on-going coordination of the validation study and/or independent peer review regarding the following seven test methods: 1) EpiSensA for skin sensitization testing, 2) α-Sens for skin sensitization testing, 3) IL-2 Luc LTT for *in vitro* immunotoxicity, 4) IL-1β Luc assay for *in vitro* immunotoxicity, 5) PyroMAT for pyrogenicity test, 6) MylcMAT for pyrogenicity test, and 7) LabCyte epithelium model-Skin irritation resting for the medical devise. Through these activities, JaCVAMhas played an active role in developing alternative methods to animal testing in safety evaluation of chemicals and quality assurance of pharmaceutical and medical devise.

Presentation: Oral

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Korea Information Center for the 3Rs: The first 12 years

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The Three Rs principles of Russell and Burch have been guiding animal use for more than 60 years. There are many 3Rs organisations that work to replace, reduce, and refine animal use and create platforms for 3Rs-related resources worldwide. Most of these organisations communicate in English. It has been an eventful 12 years since the establishment of the Korea Information Center for the 3Rs, which has the goal of facilitating the exchange of knowledge and sharing examples of good practices, and encouraging the use of alternative methods that reduce or replace animal use or to enhance humane science by avoiding or minimising pain, distress to those animals that are used in research, testing, and teaching. This presentation will summarise the progress made, and challenges faced by the Korea Information Center for the 3Rs in providing resources and educational training to promote the Three Rs, including new Korean resources for non-scientific members involved in ethical review. The collective power with international and national experts can be used to leverage an ongoing commitment aiming to provide information, resources, and practical guidelines on the Three Rs principles. It is time to plan Three Rs projects which foster interest and expertise among the next generation, including grant awards to support the development and dissemination of good practices for promoting alternative methods, high-quality science and advanced educational training programs for achieving good standards of laboratory animal welfare in Korea.

Presentation: Oral

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Evidence for refined mouse handling: Better for animals, people & science

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Traditionally, research mice are picked up by the tail (tail handling) for general husbandry or prior to conducting scientific procedures. However, a growing body of scientific literature indicates that mice find tail handling stressful, which negatively impacts mouse welfare and has behavioural and physiological consequences. To address this, an effective refinement can be implemented by instead picking up mice using a tunnel or cupped hands (refined handling).

Our practical experience of supporting the UK research community with non-aversive mouse handling methods has shown us the importance of addressing knowledge gaps for facilitating an openness to change and effective implementation. Therefore, our objective was to review and summarise the research on refined mouse handling to improve the accessibility of the findings and facilitate dissemination.

In this project, experts identified and reviewed 23 peer-reviewed empirical research articles related to refined mouse handling. Noting that the studies investigated multiple outcomes, the articles showed that refined handling improves mouse welfare (behavioural measures, n = 13; physiological measures, n = 4), voluntary interaction (n = 11), test reliability (n = 2) and breeding outcomes (n = 1). Furthermore, five articles show that refined handling is beneficial even in combination with standard procedures.

In conclusion, after reviewing the research-to-date, there are clear scientific, handler, and animal welfare benefits of using refined methods to pick up mice. Additionally, refined handling can be practical as it is compatible with common procedures and takes little additional time to implement. This review provides strong evidence for institutions considering switching to refined methods for picking up mice.

Presentation: Oral

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A novel scoring system for humane endpoints in mouse cecal ligation and puncture-induced sepsis

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Animal models of sepsis often experience high mortality due to difficulty with the prediction of death based on pre-mortem analysis and to replicate the lethality observed in human medicine. Death as an endpoint does not align with minimizing animal pain and distress. The objective of this study was to establish a scoring system for mice undergoing cecal ligation and puncture (CLP)-induced sepsis, based on visual parameters of respiratory status, activity and response to stimulus, and eye appearance. First, we evaluated the scoring system's interobserver agreement. Observations conducted between veterinary and investigative staff included 283 post-CLP mice and demonstrated near-perfect agreement (kappa > 0.81) by weighted Cohen's kappa statistic; respiratory parameter kappa-score = 0.8161, activity-stimulus kappa-score = 0.8964, and eyes kappa-score = 0.8072. The second study aim assessed the ability of the scoring system and temperature to predict mortality. C57BL/6J mice (n = 80) were monitored until death or up to 7-days post-CLP with the scoring system and subcutaneous transponders. Results showed that the scoring system discriminates between surviving and non-surviving septic mice. The scoring system demonstrated accuracy in predicting mortality, with an AUC of 0.8997 and highest sensitivity and specificity for the activity-stimulus (sensitivity: 96.30%, specificity: 92.31%) and eyes (sensitivity: 94.44%, specificity: 73.08%) parameters. Temperature represented a quantitative predictor (sensitivity: 92.60%, specificity: 92.31%). Retrospective analysis indicated that combined activity-stimulus and eye scores of 5 or greater predict death. Our scoring system demonstrates generalizability through the high interobserver agreement. Furthermore, this scoring system represents a refinement and can be implemented to determine humane endpoints for post-CLP mice.

Presentation: Oral

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Developmental toxicity test using human iPS cells based on signal disruptions induced by chemical substances

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The number of man-made chemicals including medicines has increased exponentially recently, and exposure to some of them is possible to induce fetal malformations. Although the toxicities of chemicals have been tested in animals, chemicals that are not teratogenic in rodents can cause severe malformations in humans, owing to the differences in the susceptibility to the teratogenicity of chemicals among species. One possible cause of such species differences, other than pharmacokinetics, could be the difference in sensitivity to such chemicals at the cellular level. Therefore, a human cell-based *in vitro* assay is needed for detecting potential teratogenic chemicals. Intra- and inter- cellular signaling pathways play important roles in both developmental processes and their robustness from intrinsic and extrinsic noises. We hypothesized that the developmental toxicity itself means a result that the signalings were disrupted regardless of the causes. Then, we established a signal reporter assay system based on monitoring and time-accumulation of the signal disruption over time, rather than the classical endpoint detection of the signal disruption. This approach was useful for detecting signal disruptions caused by the malformation chemicals, including thalidomide. The human iPSC-based signal disruption assay could be a promising tool for the initial screening of developmental toxicants.

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Presentation: Oral

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Why are we at a turning point in implementing and accepting nonanimal testing for vaccines batch release testing? Successes and remaining global challenges in the field

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Before they can be released to the marked, vaccine must be tested to assess the product's safety, toxicity, immunogenicity and potency. This is a fundamental regulatory requirement in place to ensure vaccines are effective and safe for use. Many tests used in batch release still rely on animals, in particular in the case of very old products like the Tetanus, Diphtheria, Pertussis, Rabies and Polio vaccines. Those tests consume large number of animals and suffer from an inherent variability that causes unexpected failures and forced retesting. Such forced test repetitions are relatively common occurrences, sacrificing additional animals and, critically, increasing costs and time needed to release the product the market.

In the last decade vaccine manufacturers in both developed and developing countries have invested in non-animal-based innovation for batch release testing with successful results. Consequently, a host of regulatory authorities have updated their requirements – or are evaluating such updates – to accommodate the innovations developed.

This presentation surveys successful examples of non-animal tests already accepted and implemented for human and veterinary vaccines, clarifying the reasons that has brought us collectively to a turning point for the local, regional and global implementation of non-animal methods and their regulatory acceptance. The presentation will then move to the remaining open technical, business, policy and cultural challenges and how those might need different approaches to be solved depending on where the solution should be implemented. A new project focused on supporting the creation of local replacement implementation plans will be presented.

Presentation: Oral

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Refining the endpoint for diphtheria and tetanus toxoid potency assays

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Lethal challenge assays are still performed to assess the potency of Diphtheria and Tetanus Toxoids in a pediatric combination vaccine for lot release and stability monitoring for the European market. These assays require multiple dilutions of the Test Vaccine and International Reference Standard to be injected into groups of guinea pigs followed by toxin challenge, with potency of the Test vaccine expressed in International Units. Although alternate serological assays are described in the Ph.Eur., the assays still require the use of a calibrated Reference vaccine and the multi-dilution design implies the use of large number of animals. In order to replace the current lethal challenge potency assays with a refined method alleviating pain and distress to animals and reducing their number in each assay, we developed an in-house single-dilution serological method with Geometric Mean Unitage (GMU) antibody titer for reporting results for Diphtheria and Tetanus Toxoid potency. Product-specific acceptance criteria were established using GMU data from 35 commercial lots. This method uses the same Test vaccine dilution to assess the immunogenicity of both Diphtheria and Tetanus Toxoids and does not require a Reference vaccine; an in-house positive control vaccine is included to confirm test validity. The assay was shown to be capable of detecting mock-prepared sub-potent vaccine batches. This new method assesses lot-to-lot consistency and allows for the refinement of the lethal endpoint. Implementation of this method will result in a significant reduction in animal usage, cost, invalidity rate and test cycle time, ensuring more robust market supply to customers.

Presentation: Oral

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Human cell-based *in vitro* systems to assess respiratory toxicity of liquid and aerosolized surfactants

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Inhalation is a major route through which substances can exert toxic effects in humans. The INSPiRE Initiative (*IN vitro* System to Predict REspiratory toxicity) aims at gaining scientific confidence in non-animal methods to assess inhalation toxicity. Recently, we have demonstrated their use for exposure to vaporized silanes. In this study, human bronchial epithelial cell line (BEAS-2B) and a reconstructed tissue model (MucilAir™) grown at an air-liquid interface will be used to show their potential to predict toxicity of another chemical class – surfactants – and to compare aerosol exposure and liquid application (pipetting).

The two cell systems, BEAS-2B and MucilAir™, were exposed to five different concentrations of Triton X-100 or oleoylsarcosine. Exposures were done by either pipetting the surfactants as liquids or by aerosol exposures using a VITROCELL® 6/4 system. Functional and cell health endpoints were assessed 19-24 hours (BEAS-2B and MucilAir™) or seven days (MucilAir™) after exposure.

Concentration-dependent effects were observed in either cell system and with both exposure methods. In addition, BEAS-2B were generally more susceptible to damage than MucilAir™ tissues.

After study completion, data from two chemical classes (silanes and surfactants), different exposure types (vapor, aerosol, liquid application), and different cell systems (cell line, reconstructed human lung tissue) will be available. The results of this project can be used to identify the usefulness of non-animal inhalation toxicity testing methods to inform regulatory decision-making as well as remaining gaps for their regulatory application.

Presentation: Oral

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Reducing the burden on animals and the heart: An evidence-based approach to cardiotoxicity assessment

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Cardiovascular disease, which is the leading cause of mortality worldwide, is suggested to be significantly exacerbated by environmental chemicals. However, cardiotoxicity is not addressed as a specific endpoint in current regulatory guidelines for chemicals, biocides, and pesticides, and assessment is heavily based on animal testing.

Within the EU-H2020 Project ALTERNATIVE (grant #101037090), we aim to improve regulatory assessment of cardiotoxicity by (1) identifying current regulatory limitations, (2) collecting and integrating multiple lines of evidence for heart failure caused by environmental chemicals using the Adverse Outcome Pathway (AOP) framework, (3) evaluating the weight of evidence according to OECD guidance, (4) identifying suitable non-animal methods and Biomarkers of Effect (BoEs) to assess key events of the AOP, and (5) drafting an Integrated Approach to Testing and Assessment (IATA).

We identified different gaps in cardiotoxicity assessment, low predictivity of animal-based as well as *in vitro* cardiotoxicity methods used in current regulatory guidelines, and the need to address the elderly population and mixtures. We developed a novel AOP network (including AOPs #479 & #480) that causally combines and integrates epidemiological human health evidence (15 studies), as well as experimental *in vivo*, and *in vitro* evidence (360 studies) from our systematic reviews on heart failure caused by environmental chemicals. The weight of evidence is evaluated using OECD guidance and omics analysis will be used to validate key events and identify corresponding BoEs.

These AOPs will ultimately provide a foundation to an IATA for organ toxicity, including cardiotoxicity, and reduce the dependence on animal testing.

Presentation: Oral

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Towards a mechanistic characterization of metabolic disruption using proteomics

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The pandemic development of obesity is presumed to be accelerated by endocrine disruptors such as plasticizers and bisphenol A (BPA). Understanding the modes of action (MoAs) underlying metabolic disruption is crucial to establish non-animal methods for evaluation of endocrine disruptors.

To increase our understanding of metabolic disruptive effects in adipose tissue, we examined the effects of 25 chemicals, including BPA analogues and plasticizers, on two key cell types: human adipocytes and macrophages. Our study examined molecular initiation of the master regulator of adipogenesis, the peroxisome proliferator-activated receptor gamma (PPARγ). Using *in vitro* assays and global proteomics, we characterized altered pathways, biomarkers, and functionality.

Our results revealed that BPA and its substitutes inhibited PPARγ, leading to inhibition of adipogenesis, an inflammatory state, and impaired insulin sensitivity. In contrast, plasticizer metabolites induced adipogenesis in preadipocytes via PPARγ activation, displaying similarities and overlapping key drivers with the PPARγ agonist rosiglitazone as assessed by Weighted Gene Correlation Network Analysis (WGCNA). Plasticizers and their metabolites caused oxidative stress and adipocyte dysfunction in mature adipocytes. In addition, the most potent obesogenic plasticizer exacerbated oxidative stress and hence the immune response in macrophages.

Consequently, these metabolic disruptors may promote hypertrophic dysfunction and chronic adipose tissue inflammation, contributing to metabolically unhealthy obesity and associated complications. Integrating the mechanistic knowledge into an adverse outcome pathway (AOP) network may provide a roadmap for regulatory assessment without the need for animal testing. The key to unlocking this knowledge is a deeper understanding of the MoAs, which can be achieved through omics.

Presentation: Oral

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OrbiTox: An interactive predictive toxicology and translational discovery tool

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As the reliance on traditional animal testing phases out, increasing use of new approach methodologies such as *in vitro* assays and *in-silico* methods are generating massive amount of data which are often difficult to utilize efficiently. OrbiTox addresses this challenge by providing an interactive visualization for a collective view of "bigdata" from different domains (chemicals, genes, pathways, organisms) and informative connectivity across them to support translational discovery. OrbiTox currently houses curated data from ~900,000 substances, ~22,000 annotated human targets, ~1,500 biological pathways, and over 100 test organisms. There are also more than 400,000 connections representing experimental, curated human and rodent carcinogenicity, bacterial mutagenicity, acute toxicity and various bioactivities data. Additionally, OrbiTox offers over 100 novel QSAR models that provide chemistry-backed reasoning for predictions in 80 Tox21 assays, bacterial mutagenicity in OECD-recommended strains, and cardio and ocular toxicity. Unique 3D organization of high-dimensional multi-domain data connected in concentric 3D-globes results in an intuitive and inviting environment enabling queries from any data domain. For a query chemical, one can access experimental and predicted chemical profiles and identify structural analogues using Saagar substructures (PMID: 33356152). In one test study, we were able to query the fructose metabolism pathway, identify its member genes, identify inhibitors of its member gene AKR1B1 with their IC50 values – all in one view. The unique functionality of OrbiTox to map complete paths between multiple query objects is extension of Swanson's ABC inference paradigm of open discovery process to generate hypotheses for functional or mechanistic relationships between searched entities.

Presentation: Oral

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Alternative methods for potency and safety test for batch release in human vaccines

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Vaccines contribute to improved health and welfare of humans by preventing and controlling infectious diseases agents that can cause disease and death. However, vaccine batches require quality assessment before being released to the market and in many cases these tests are *in vivo*. This requires a significant number of animals to ensure vaccine effectiveness and safety and can lead to significant pain and distress.

Currently, *in vivo* potency tests are being used in the quality control of human vaccine testing for each final bulk or lot before release onto the market. This is particularly true for traditional vaccines like Diphtheria, Pertussis, Tetanus, Rabies, Hepatitis B and Hepatitis A and Polio vaccines. In the last decades, many studies have been conducted to validate alternative methods for quality control and batch release especially for potency and safety test however harmonization of guidelines is required to implement these alternative approaches.

Now the time has come to substitute or reduce animal use for potency testing. There is strong scientific evidence that *In vitro* tests, are sufficient to detect differences that are relevant to the control of the production process. Similarly, for safety testing, *In vitro* assays, mainly cell culture assays, should be used. These approaches are more sensitive and reproducible when compared to *In vivo* tests.

Based on current good manufacturing practices, process validation, in-process testing, validated analytical testing and the consistency approach, vaccine quality, safety and efficacy can be demonstrated without routine use of *In vivo* tests.

Presentation: Oral

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Bird's eye view: Strengthening protections for wild animals in research

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Research on wildlife is understudied and underregulated. With more than 73,000 wild vertebrates, federal guidelines cannot address the topics critical for wildlife research. Most Institutional Animal Care and Use Committees do not include wildlife researchers nor is there sufficient research or updated guidelines to refer to during the research review process. Wild species are brought to the laboratory without adequate protection and their welfare cannot be granted due to lack of species-specific knowledge. We have found several cases where animals have died under the care of the investigator, both accidentally due to ignorance surrounding animal care, and purposefully as part of the experiment. By reviewing U.S. wildlife permit protocols from 2019-2022, we aim to collect information to understand the use of wild animals in research and use it to foster effective protections for species subjected to experimentation. The purpose for wildlife permits varies, animals are sometimes collected to study diabetes, cancer, and biomechanics research, in some cases with the intention to inform human health. With inadequate protection and insufficient research to recognize causes of distress, negative reactions to change of diet, humane handling, etc., holding them captive in labs is an ethical concern. Through state-by-state monitoring, we are working to understand the use of wild animals in research and establish protections for them. Each state has different requirements for permit approval, but we anticipate the information gathered will lead to changes in policy recommendations at the institutional, state, and federal levels.

Presentation: Oral

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EDQM's work on implementing alternatives to animal testing

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Since the 1980s, with the elaboration of European Convention (ETS 123) for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, the European Directorate for the Quality of Medicines and HealthCare (EDQM) of the Council of Europe (COE) has been committed to 3Rs. Activities focus on inclusion of non-animal tests, removal of obsolete animal tests as well as reduction and refinement of existing animal tests for both human and veterinary biologicals, including vaccines. These changes are included for adoption in the European Pharmacopoeia wherever scientifically justified. This work has led for example to the deletion of the abnormal toxicity test (ATT), specific toxicity tests (diphtheria (D) and tetanus (T) vaccines) and the irreversibility tests for T and acellular pertussis (aP) vaccines as well as the inclusion of *in vitro* or serological based potency assays in different contexts e.g. D,T, aP, hepatitis B vaccines. Chap-

ter 5.2.14 importantly provides guidance on how to substitute *in vivo* methods with *in vitro* ones to facilitate *in vitro* approach implementation, highlighting the scientific benefits of the *in vitro* approach and where there is lack of justification for direct correlation between *in vitro* and *in vivo* tests. An important ongoing endeavour is the full replacement of the Rabbit Pyrogen Test (RPT). The presentation will highlight the path that EDQM has taken towards the implementation of non-animal testing with a focus on the ongoing deletion of RPT from the pharmacopoeial requirements and its replacement with other approaches and testing methodologies.

Presentation: Oral

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Inference model performance: A key component for scientific confidence frameworks for NAMs

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A NAM must meet a requisite degree of scientific confidence for one or more specific applications before it can be used to support product stewardship or regulatory decisions. While various components of "fit for purpose validation" (FFPV) have been discussed, a uniform approach for FFPV has yet to be adopted. Accordingly, we proposed a scientifically rigorous, yet flexible framework, that can be widely applied to most types of NAMs. This NAM Scientific Confidence Framework (SCF), centered on explicitly applying the scientific method, consists of 7 components: 1) problem formulation and hypothesis (explicit proposition that the NAM can be used to provide actionable information for a specific decision context) 2) the biological relevance & plausibility of the NAM; 3) assay performance (documentation of sensitivity, specificity, reliability, & domain of applicability of the NAM); 4) documentation of the performance of inference (prediction) models based on the NAM response – outcome response relationship; 5) dissemination of the data, inference models, etc. to support independent replication; 6) a narrative rationale making the case that there is / is not sufficient scientific confidence in the NAM to support the specific application for the chemistry domain of interest; and 7) verification through independent scientific peer review. While biological relevance is necessary, it is not sufficient; documenting inference model performance is needed. We will illustrate application of this NAM SCF, focusing on inference model performance, to the evaluation of the key characteristics of carcinogens, to TTCs and points of departure from ToxCast data, and to an AOP.

Presentation: Oral

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Regulatory considerations for the development, licensure, and use of non-animal-based quality control assays for vaccines

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The development of a well-defined manufacturing process and assays to ensure vaccine quality and safety are critical for the licensure of vaccines that will be safe, pure, and potent. The selection and validation of quality control assays is based on the best technology available at the time of vaccine development. Historically, animal-based assays were developed to measure vaccine safety and potency. While these assays may have been appropriate at the time of development, recent advances in analytical technologies and our understanding of the critical attributes of vaccine products has led to the development of alternative assays that can replace the animal-based assays that were used at the time of product licensure. This presentation will highlight regulatory considerations related to the development of alternative assays and provide some examples where new assays are being implemented to replace existing animal-based assays currently being used to assure product safety with respect to adventitious agents testing.

Presentation: Oral

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In the room where it happens: Assessing pesticide hazard

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In vivo studies with laboratory animals are required and/or used to evaluate pesticide exposures; however, regulatory statutes provide the United States Environmental Protection Agency (U.S. EPA) with the flexibility to modify the actual data and studies required on an individual chemical basis. Therefore, the Agency may use data from alternative methods and strategies to satisfy data requirements. The U.S. EPA Office of Pesticide Programs (OPP) has been working with multiple national and international organizations to reduce its reliance on animal testing through the development and implementation of new approach methodologies (NAMs) for regulatory purposes. To put ongoing NAM efforts into context, this presentation will provide an overview of how U.S. EPA assesses

pesticide hazard. This will include discussion of the process taken by OPP to evaluate and interpret an extensive amount of data from toxicological studies, which provide the agency with information on a wide range of adverse health outcomes, different routes of exposures, varying durations, species differences, and life stage information, to support pesticide registration.

Presentation: Oral

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A step-by-step approach for assessing acute oral toxicity without animal testing for cosmetic ingredients

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Animal testing of cosmetic ingredients and products has been banned in the European Union since 2013. However, the safety evaluation of a cosmetic ingredient requires the generation of data on acute oral toxicity through animal testing. In the present study, we developed the step-by-step approach for assessing the applicability of acute oral toxicity for cosmetic ingredients without animal testing. This approach was challenged in the Weight of Evidence (WoE) assessments of acute oral toxicity using a combination of safety data, including a neutral red uptake cytotoxicity assay using BALB/c3T3 cells (3T3-NRU cytotoxicity assay) that can assess the acute oral toxicity for additives of quasi-drugs or cosmetic ingredients. However, our approach is out of scope for major ingredients of quasi-drugs and a request for revision of cosmetic standards. We conclude that the step-by-step approach can be used to assess test substances that cause low acute oral toxicity within this applicability domain, such as the median lethal dose (LD 50) > 2000 mg/kg based on our proposal, thereby avoiding animal testing.

Presentation: Oral

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Latest activities and future directions of JSAAE for Asian cooperation toward 3Rs

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After the official establishment in 1990, the Japanese Society for Alternatives to Animal Experiments (JSAAE) has grown to have about 400 members as of 2023. While promoting 3Rs research in Japan through a wide variety of domestic activities, we have been making serious efforts to various aspects of international cooperation and contributions to 3Rs, such as hosting 6th Congress on Alternatives and Animal Use in the Life Sciences (WC6: 2007) in Tokyo. JSAAE now have cooperation agreements with the similar societies among EU (European Society for Alternatives To Animal Testing: EUSAAT and European Society of Toxicology in Vitro: ESTIV) and USA (American Society for Cellular and Computational Toxicology: ASCCT).

In addition, the Korean Society for Alternatives to Animal Experiments (KSAAE) recently organized 3rd Asian Congress for Alternatives to Animal Experiments (ACAAE), December 2022 in Jeju, Korea. The International cooperation is taking root in Asia definitely. As a next step, we are also planning to set up the Asian Federation with KSAAE, Chinese Toxicological Alternatives and Translational Toxicology (TATT) and Society for Alternatives to Animal Experiments-Indian (SAAE-I), where the concept of the 3 Rs is just now achieving penetration.

Presentation: Oral

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3D spheroids of the pancreatic beta cell line EndoC-βH5 for modelling diabetes-on-chip

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In Type 2 diabetes mellitus (T2DM), glucose homeostasis is impaired. To date, this multi-organ disease cannot adequately be reflected in animal models or standard *in vitro* models. To address this challenge, we have recently shown functional coupling of human HepaRG spheroids and human islet microtissues for studying the interplay between the pancreas and the liver, two key organs in blood glucose regulation [1]. Here we show spheroids made of EndoC-βH5 cells as an alternative pancreas organ model.

The human pancreatic β-cell line EndoC-βH5 exhibits a near-native β-cell phenotype and is available as ready-to-use frozen stocks. We first optimized a protocol for spheroid formation and observed stable morphology and stable glucose-stimulated insulin secretion (GSIS). The EndoC-βH5 spheroids showed dose-dependent insulin response to glucose and, moreover, stimulation with the GLP-1 receptor analog exenatide led to a significantly increased insulin secretion. We then demonstrated that the EndoC-βH5 spheroid model can be co-cultivated together with HepaRG spheroids in the HUMIMIC Chip2 for up to 15 days. Liver and EndoC-βH5 cell functionality, as shown by albumin and insulin secretion, respectively, was stable over time. The liver-pancreas chip results were translated to computer models, which allowed us to test mechanistic hypotheses, and to scale volumes and add additional organs in the computer.

The newly developed 3D spheroid model of EndoC-βH5 cells represents a reproducible and functional human β-cell model to study the liver-pancreas axis *in vitro*, which can be used of new therapies.

Reference

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Presentation: Oral

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The path to better science: 3Rs and more

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It is widely accepted that there is still great room for improvement when planning and conducting experiments which appear to involve the use of animals. There are good scientific and ethical reasons for working to advance the three Rs: Replacement, Reduction and Refinement. In addition, we must ensure that those experiments which still have to use animals are correctly designed, so that the results are transferable to other animals and to humans.

The path to better science is long and involves good preparation from day 1, starting with an evaluation of the possibility of using alternatives to animal experiments. These efforts must involve specialists in literature searches, experts within non-animal methodologies and, if animal studies are unavoidable, close collaboration with animal care staff who know the strengths and limitations of the facility.

To give scientists an overview of all the factors which can influence the validity and outcomes of animal research, we have developed a set of guidelines called PREPARE, based upon experiences in managing accredited animal facilities. PREPARE consists of a two-page checklist, available in over 30 languages, and a comprehensive website with links to more information and the latest resources about each topic on the checklist (https://norecopa.no/PREPARE).

To encourage advances in animal research, we have also created a Refinement Wiki, which can be used to publish large or small improvements to housing, care and use (https://wiki.norecopa.no).

Together we can PREPARE for, and achieve, better Science.

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Development towards an organ-on-the-chip technology for complex *in vitro* testing of T cell products

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The clinical development of advanced human cell therapies suffers from a lack of adequate preclinical testing in laboratory animals. The informative value of such (humanized) animal trials are limited due to their phylogenetic distance to humans and, especially, their lack of a human immune system. Due to the histocompatibility mismatch between laboratory animals and the patient, challenges increase significantly once personalized T cell therapy approaches are under evaluation.

Here, we used the HUMIMIC® multi-organ-chip platform to establish a human *in vitro* rejection assay using iPSC-derived endothelial cells, iPSC-derived kidney organoids, and PBMCs. The platform enables co-culture of different organ models but lacks blood micro-capillary vessel structures covered with human endothelial cells. For this purpose, we implemented a network of miniature vascularized channels in the organ compartments of the HUMIMIC® platform for capillarization of organ models exploring 3D printing tools and endothelial self-assembly processes. Organ models were generated from iPSCs of two different individual HLA-tested healthy persons emulating the recipient and the donor background. Multi-organ-chip design and prototyping results are presented along with on-chip micro-vessel formation and culture over prolonged culture periods. Furthermore, we present data of establishing the rejection assay, statically and dynamically. Results will be discussed in the light of the assay potential to test immunosuppressive drugs and cell therapies (e.g., transplant rejection) to replace respective animal models in use.

Presentation: Oral

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The consistency approach for the substitution of *in vivo* testing for the quality control of established vaccines: Practical considerations and progressive vision

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This presentation summarizes the discussions and proposals made by the VAC2VAC consortium on how to deploy the "Consistency Approach" for quality control of established vaccines and thus facilitate the substitution of *in vivo* testing. This work answers specific questions about "what does a control strategy according to the consistency testing look like" and "how to submit a control strategy defined according to the consistency testing".

Some topics were answered in a very straightforward manner. This was the case when the deployment of the consistency approach and the corresponding changes in vaccines control strategy was supported by the generic application of procedures already described in regulatory guidelines/requirements and related to the establishment or change in the control strategy of vaccines. The application of other procedures required more specific attention to reach a proposal.

The key outcomes of this work are that robust science must be used to develop a substitution strategy and generate supportive data packages. And this good science can best occur with good scientific collaboration between the different parties involved. Early interaction between manufacturers and authorities before and during dossier submission is critical to success. The consistency approach, when approved and in place, will ensure vaccine products of assured quality reach the patient more efficiently than when relying on animal testing. Adapting the mindset was one of the major hurdles to a progressive vision but there is now consensus between manufacturers and competent authorities to foster the elimination of animal testing for routine vaccine release testing.

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Non-animal methods in quality control of vaccine testing batch release

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When using animals, we follow scientific principles to ensure that we prevent or minimize pain and distress before, during, and after experimental procedures to develop innovative products and perform the mandatory release of vaccines. Historically, the majority of animal testing in GSK is linked to quality control of vaccines making it a key element to reduce animal use by developing and applying non-animal methods (NAM). GSK has reduced the utilization of animals by 60% in the last seven years and we aim for 75% by 2025 in Vaccines Quality.

Our 3R strategy is to develop and validate NAM for routine testing to assess the safety and potency of vaccines in release and stability analysis. This is achieved by internal activities and external collaborations to co-build assays that align industry and authority requirements. While potency assays allow reduction by statistical improvements, safety assays do require the development of NAM which are expected to be superior in their critical quality attributes. GSK has achieved this by reducing the amount of requested repetitive *in vivo* safety testing in different franchises such as Tetanus. In addition, the development of product tailored assays with improved sensitivity for pyrogen detection and the safety assessment of specific products such as polio vaccine, have reduced the animal testing. The qualified NAM have proven to be more robust and faster while ensuring the safety of the patient. Besides the ethical gains, our 3R activities allow to release vaccines faster, with less variable methods and in a leaner procedure.

Presentation: Oral

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Replicability of preclinical cancer biology

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Replicability is an important feature of scientific research, but aspects of contemporary research culture, such as an emphasis on novelty, can make replicability seem less important than it should be. This presentation will describe the results of an 8-year project to repeat 193 experiments from 53 high-impact papers in preclinical cancer biology, using an approach in which the experimental protocols and plans for data analysis had to be peer reviewed and accepted for publication before experimental work could begin. Various barriers and challenges were encountered while designing and conducting the experiments limiting the number of experiments that could be conducted and analyzed. In the end, a total of 50 experiments from 23 papers were repeated, generating data about the replicability of a total of 158 effects. The median effect size in the replications was 85% smaller than the median effect size in the original experiments. When looking at a number of binary methods – that is, the replication was determined to be either a success or a failure – the overall replication success rate was 46% (51/112). A successful replication does not definitively confirm an original finding or its theoretical interpretation. Equally, a failure to replicate does not disconfirm a finding, but it does suggest that additional investigation is needed to establish its reliability. Overall, this experience draws attention to a basic and fundamental concern about replication – it is hard to assess whether reported findings are credible.

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ICE data and tools to advance NAMs

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The Integrated Chemical Environment (ICE, https://ice.ntp.niehs.nih.gov/) provides highly curated toxicologically relevant data and analytical tools for data interpretation and exploration. ICE has made significant contributions to advancing NAMs, with data and tools being continuously updated to address evolving stakeholder needs. ICE version 4.0, released in March 2023, is a major update. ICE users can now obtain general population-level exposure predictions from EPA's SEEM3 model across multiple scenarios through the ICE Search tool and the ICE Search API. Exposure estimates can also be compared to the equivalent administered doses predicted by the ICE *In Vitro* to *In Vivo* Extrapolation (IVIVE) tool. To better support development of new methods for developmental toxicity, ICE v.4.0 adds a new gestational model from the EPA's httk package to its Physiologically Based Pharmacokinetic (PBPK) and IVIVE tools. The ICE curated high-throughput screening (cHTS) dataset has been updated with the latest ToxCast and Tox21 data, providing *in vitro* data to use in bioactivity as-

sessments and computational toxicology workflows. Several new warning flags for autofluorescence interference have been added into the curation workflow. Updates to the underlying chemical use data in the ICE Chemical Characterization tool have also been implemented, with reported and predicted functional use categories now available in addition to consumer use data. The presentation will include case studies to demonstrate how ICE can facilitate building confidence in NAM use for chemical assessments.

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Presentation: Oral

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Deep learning profile QSAR modeling to impute *in vitro* assay results and predict chemical carcinogenesis mechanisms

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Carcinogenesis is a multistep process in which normal cells acquire various properties that allow them to form benign tumors or malignant cancers. These properties of cells have been associated with 10 well-established hallmarks of cancer (HMC). It has been further suggested that human carcinogens (e.g., chemicals, viruses) share one or more of 10 properties, namely key characteristics of carcinogens (KCC). QSAR models that rely on structural and/or physicochemical properties to predict carcinogenesis potential endpoints usually exhibit low performance, likely because they lack sufficient information on the complex mechanisms involved in carcinogenicity. We used a novel imputation profile QSAR modeling approach coupled with deep learning to analyze data on 10,000 Tox21/ToxCast chemicals and 2,000 *in vitro* assay endpoints subsetted by HMC and KCC. Because limited experimental data were available, we filled data gaps by imputing assay results for the Tox21/ToxCast inventory using structural and physicochemical properties and deep learning. *In vitro* assay results were enriched using data in the BioBricks platform (https://biobricks.ai/bricks/), which compiles toxicity-relevant databases into a harmonized easily accessible format. This enrichment allowed us to include additional information such as protein target binding in the model. Finally, multitask deep learning was applied to predict each chemical's likelihood of triggering cancer HMC and KCC based on the imputed and enriched *in vitro* data. Results included data on the quality of imputation, defined by grouping of assays, and performance computed per chemical.

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Presentation: Oral

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Human neuron-on-a-chip platform to automate the screening of compounds targeting Alzheimer's disease

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There is no cure and no efficient therapy for most neurological disorders, including Alzheimer's Disease (AD), a progressive type of dementia largely associated with the loss of synapses in the brain. Today, 99.6% of the drugs targeting neurological diseases that pass animal tests fail on human trials and currently available animal models fail to reproduce the pathological complexities of AD. For the successful development of new therapies, robust alternative approaches methods (NAMs) that specifically recapitulates the human neuronal signaling pathways are required to elucidate the mechanism of neurodegeneration in AD. Ananda Devices developed technology for rapid growth and precise organization of human neuronal networks-on-a-chip [1,2] in scalable multi-well microplates, enabling automation of the analysis of multiple neuronal parameters in 3000 individual neurons per plate (NeuroHTS™ platform). In this study, we used the NeuroHTS™ platform to compare neuronal morphology, network dynamics, synapses, and electrical function of excitatory neurons derived from human induced pluripotent cells from healthy and AD donors. Results showed key differences in axonal growth, axonal thickness, neuronal connections, synapse formation, synaptic maturity, and tau distribution. Next, we used the NeuroHTS™ platform to compare the effects of 4 different potential therapeutic compounds (Aptah Bio Inc.) in reducing degeneration of neurons from AD donors. The results were validated using RNAseq analysis pointing to key signaling pathways involved in neuro-

degeneration. The data highlight how Ananda Devices' human based, *in vitro*, NeuroHTS™ platform enables rapid and automated compound screening directly on patient's derived cells.

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Presentation: Oral

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Rethinking NAM adversity

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New Approach Methods (NAMs) provide information on how substances can perturb signaling and lead to adverse outcomes and are faster, higher throughput, less expensive, reduce the need for animal testing, and can be designed to be more biologically relevant than traditional animal tests. NAMs can support assessment of chemical effects on human and environmental health; however, it may be a challenge to define what constitutes "adversity". To date, adversity based on NAM data has been established by the correct prediction of the effects of positive/negative reference chemicals identified from animal tests. Chemical databases facilitate identification of such chemicals; however, the reference chemicals may become associated with the guideline in which they were tested rather than the affected endpoint. In recent analyses of multigeneration reproduction and prenatal development studies in the US EPA's ToxRefDB, non-specific endpoints (e.g., liver or body weight) were altered at the critical effect level in ~90% of studies. Additionally, < 20% of 9800 chemicals in the REACH database were associated with any specific type of toxicity. While chemical safety decisions based on (any) effects observed in animals may be protective of human health, these chemicals do not necessarily exert the specific adverse response associated with the *in vivo* test. The misconception of what "positive" effects these chemicals have *in vivo* is transmitted to the evaluation of the ability of NAMs to predict adversity. Developing NAMs that are protective of non-specific *in vivo* effects would align with how animal test data are often used in regulatory decision making.

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OECD path to mutual acceptance of NAM data

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The OECD system of Mutual Acceptance of Data (MAD) is an agreement among countries to accept results from OECD Test Guidelines conducted in labs following Good Laboratory Practices. MAD avoids duplicating data and saves > 300M €/year in testing costs and 10,000s of lab animals. Using New Approach Methods (NAMs) in regulatory risk assessments creates opportunities to increase chemical safety information, while decreasing costs and animal use. However, international chemical legislations differ regarding specific data requirements and flexibility to fulfil requirements, and thus national initiatives to use NAMs in chemical assessment potentially erode the benefits of MAD. Recent experiences using NAMs for evaluating chemical effects on humans led to efforts to develop guidance for the use of non-guideline data and non-binding criteria for building confidence in innovative methods. These experiences facilitated the use of NAM Test Guidelines (e.g., Defined Approaches) and standardization of methods that are not (yet) proposed for inclusion in Test Guidelines (e.g., omics, PBK model reporting, etc.). The OECD Chemical Programme is leveraging lessons learned from developing human health related NAMs for developing NAMs for ecotoxicological assessment. Though there are fewer examples of IATAs for ecotoxicology, OECD Case Studies use standardized reporting formats, include transparent reporting of uncertainties, undergo peer-review, and include a consideration for the global applicability of ecotoxicity NAMs. In addition to this foundational work, the OECD is planning a workshop to evaluate how innovative methods can contribute to protecting the environment from harmful effects of chemicals and help preserving biodiversity.

Presentation: Oral

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The OECD and the future of risk assessment

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Marking 50 years of the OECD Chemical Safety Programme created an opportunity to reflect on past milestones and future goals for international risk assessment. There are now ~35 *in vitro* methods included in OECD Test Guidelines, and the Ad-

verse Outcome Pathway Knowledgebase (AOP-KB) has provided a proof-of-concept framework for how *in vitro* methods can be used to predict *in vivo* outcomes. In addition to OECD Test Guidelines, a variety of initiatives are intended to increase implementation of modern toxicological methods in regulatory decision making. OECD harmonized data reporting templates and standards contribute to structured databases and facilitate sharing chemical safety data globally. A QSAR Assessment Framework, which includes a checklist of criteria and guidance for considering the suitability of QSAR models and predictions used in a regulatory context, will be finalized in 2023. The Integrated Approaches to Testing and Assessment (IATA) Case Studies Project, in its 9th year, has published > 35 examples demonstrating how innovative approaches can be used to assess chemical hazards, as well as general and topic-specific guidance associated with IATAs. These projects have standardized reporting, evaluation of confidence/uncertainties, peer-review, and evaluation of suitability of innovative approaches for a variety of regulatory applications. OECD endorsement of these IATAs does not mean results are accepted under the agreement on Mutual Acceptance of Data (MAD) but can lead to new OECD Test Guidelines (which are covered by MAD) and create opportunities where authorities can choose to accept not only data, but also the second "A", the assessment of chemicals.

Presentation: Oral

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Human-relevant models in biomedical research – Progressing science through innovation

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Research heavily relies on animal-based models as revealed by the latest statistics on the use of animals in the EU showing that 72% of them are employed in basic, translational and applied research. Since the ultimate aim of the Directive 2010/63/EU is the replacement of animal experimentation with alternatives, targeting this domain must be a priority.

For this reason, EURL-ECVAM performed a series of studies to investigate non-animal-based approaches used in biomedical research in seven disease areas: respiratory diseases, breast cancer, neurodegenerative diseases, immuno-oncology, immunogenicity testing for advanced medicinal therapy products, cardiovascular diseases and autoimmune diseases. For each area, several databases were searched to retrieve relevant publications. In total, 322,827 abstracts were screened, 89,446 full texts were analyzed, and 3,049 non-animal methods were selected to create highly curated and publicly available databases. The prevailing methods employed differ among research areas, however those involving cell culture were the main methods for all areas except for cardiovascular diseases, for which *in silico* models were most used. Overall, in the last few years an increase in the development and use of organ-on-chip technologies was observed, particularly for respiratory and neurodegenerative diseases.

These reviews have produced a knowledge base freely available to a variety of stakeholders and can be exploited to explore the strengths and limitations of both animal and non-animal models used in biomedical research. Most importantly, it represents an essential tool for producing evidence-based policy recommendations aimed at improving research translatability for the ultimate benefit of patients and society.

Presentation: Oral

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Increasing understanding & implementation of refined mouse handling: A longitudinal, cross-sectional benchmarking survey

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Refined mouse handling improves research quality and mouse welfare, yet widespread implementation seems to be low. We lack a clear understanding of current levels of implementation and the reasons behind them (particularly in the USA) that could be used to design interventions. The purpose of this project was to benchmark prevalence of, associations with, and beliefs towards refined mouse handling over a 3-year period.

Research personnel were recruited to complete an innovative mixed-methods longitudinal benchmarking survey grounded in the validated theory of planned behavior. Per year at least 250 participants responded, and 50 participants completed the survey all 3 years. Participants were primarily from the USA. Quantitative data were analyzed via descriptive statistics and generalized regression. Qualitative data were analyzed by theme.

Over 3 years, implementation of refined handling was low. However, intentions to provide refined handling in the future increased over time. Furthermore, this intention was strongly associated with attitudes, norms, and control beliefs (p's < 0.01). Finally, although the distribution of reported barriers changed over time, participants primarily cited misconceptions about the technique (perceived incompatibility with jumpy mice and restraint),

operational concerns (lack of time or tunnels), and other personnel. More misconceptions were reported in year 1 than 3.

In conclusion, although these results indicate that refined mouse handling and related beliefs are improving over time, they also indicate that more work is needed. Educational strategies to encourage further implementation may need to change over time from addressing core misconceptions to assisting with operational concerns and convincing others.

Presentation: Oral

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Charting the pervasive changes in alternative splicing networks to study novel modes-of-action in toxicogenomics

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Advances in genomics and computational tools have led to better acceptance of new approach methods (NAMs) as alternatives for animal testing for health assessments of chemicals and drugs. Biological endpoints and modes-of-action (MoA) can be inferred based on the changes in transcriptional profiles prior to observed changes in phenotype. Here, a transcriptomic mechanism called alternative splicing (AS) of RNA transcripts was investigated. AS involves the combination of various sequences (exons and introns) from nascent RNAs in several ways to generate a variety of RNA isoforms from a single gene. AS plays key roles in transcriptional control during cellular physiology, development, and the diversification of the proteome. In many diseases, including cancer, aberrant AS is strongly linked to disrupted cellular development, and its modulation is currently employed as a treatment method. However, a deep assessment of chemical-induced AS changes in Toxicogenomics has not previously been performed, as current studies heavily rely focusing on differential gene expression (DEG) analyses. By implementing a well-established AS analysis pipeline which quantify splicing junction RNA-seq reads and splicing events, we identify that chemicals that induce oxidative stress, such as Paraquat or Prochloraz, result in widespread changes in AS networks even at doses without observed DEGs. Altogether, we uncover a previously underappreciated molecular mechanism that can potentially explain the cellular effects of many chemicals. Here at ScitoVation, we demonstrate the latest transcriptomic computational approach for the application of the 3Rs in chemical health assessments, in particular reducing animal testing and eventual replacement with increased acceptance of NAMs.

Presentation: Oral

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The "stats" on statistics courses: Requirements in select Canadian U15 graduate programs

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Efficient experimental design and rigorous statistical analysis are important aspects of reproducible animal-based research. Recognizing that training in experimental design and statistics for research may start in graduate school, the purpose of this study was to determine the extent to which graduate courses in experimental design or statistics are required, recommended, or offered by graduate programs in animal science, neuroscience, pharmacology, and psychology at Canadian U15 universities. Data on Master's and Ph.D. program requirements were accessed through online academic calendars or equivalent official sources of program information. Relevant course offerings were identified through keyword searches of course titles and descriptions. Fewer than 15% of animal science, neuroscience, and pharmacology programs had requirements for experimental design or statistics courses, and fewer than 30% had recommendations. In contrast, up to 93% of psychology programs required these courses, and up to 47% recommended them. More than 90% of graduate programs in animal science and psychology offered courses in experimental design or statistics, whereas fewer than 35% of neuroscience and pharmacology programs had these offerings. Prerequisite course requirements or other registration restrictions were uncommon for all courses identified across disciplines. Thus, the extent to which experimental design or statistics courses feature in graduate programs of different disciplines varies, and for three of the four disciplines evaluated, graduate training in experimental design or statistics often falls outside of formal program requirements.

Funding: Natural Sciences and Engineering Research Council of Canada Undergraduate Student Research Award, Mitacs Accelerate Internship, and Canadian Council on Animal Care

Presentation: Oral

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Integrating enzyme variability into PB-K models of chemicals and metabolites

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Chemicals that enter the body are metabolized via a number of pathways. These rates of metabolism can vary across human populations due to genetic variability of metabolic enzymes, meaning some populations are more sensitive to effects of parent chemicals or metabolites. Risk assessors apply physiologically-based kinetic (PB-K) models to predict the dynamics of tissue concentrations for parent chemicals and their metabolites, but it is difficult to use these models to characterize the effects of enzymatic pathway-related variability. We developed a generalized workflow for incorporating pathway-related variability for select Phase I CYP and Phase II UGT enzymes across human populations into PB-K models. The workflow includes metabolite structures generated using SimulationsPlus ADMET Predictor®, PB-K models from EPA's httk package, estimates of inter-individual enzyme variability from EFSA literature reports, and parameter predictions from OPERA (v2.8). Parent chemical dynamics are simulated following initial exposure and the amount of parent metabolized is scaled by percent yield to provide an intravenous time series for metabolite models. Ranges of parent and metabolite concentrations are estimated by Monte Carlo sampling of enzymatic variability in intrinsic clearance. This presentation will demonstrate the dynamics of the workflow and include a case study of 10 parent chemicals and their metabolites. The workflow provides a characterization of tissue concentration dynamics for potentially toxic chemicals to support estimates of hazard and risk for sensitive subgroups of an exposed population. Results will eventually be accessible through the Integrated Chemical Environment (ICE; https://ice.ntp.niehs.nih.gov/).

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Presentation: Oral

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Building confidence in a new approach to agrochemical carcinogenicity assessment

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While the life-time rodent cancer bioassay remains the primary study design to support regulatory requirements for carcinogenicity assessment, decades of research have underscored the limitations of its use including the lack of human relevance, low throughput, high variability, and issues related to animal welfare. Given the extensive limitations, case studies are being developed to illustrate how an iterative weight-of-evidence (WoE) based approach fulfills regulatory carcinogenicity assessment requirements without performing the rat and mouse cancer bioassays. The WoE approach to carcinogenicity assessment has been shown to be health protective by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) expert workgroup and resulted in an addendum to the S1 carcinogenicity assessment guidance. This presentation will share the learnings from a pilot retrospective case study, the iterative process used to strengthen a follow-up prospective waiver, and clarification of the process to inform the development of a cluster of case studies to be submitted to the OECD IATA Case Study Project (CSP) in 2023. Revisions of the pilot and progress from prospective case studies utilizing the reviewers' guidance and recommendation will be presented to outline the critical areas of focus and opportunities to strengthen the research and development of agrochemicals. This approach must establish confidence in reducing uncertainties associated with human safety testing methods without conducting lifetime tests on animals.

Presentation: Oral

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Building value and trust through scientific rigor and transparency

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Animal research is regulated under the premise that any harm imposed on animals must be indispensable. This is also the legal basis of the 3Rs principle (replace, reduce, refine), which serves to

minimize harm to animals in research. However, the 3Rs are just one among several ethical principles needed to determine the indispensability of animal research. Ultimately, the decision is taken by ethical deliberation in a harm-benefit analysis (HBA). Yet unless study findings are valid and reproducible, animals may be harmed without producing any benefit. Sources of poor reproducibility include poorly validated animal models (poor construct validity), a lack of scientific rigor (poor internal validity), and rigorous standardization (poor external validity). I have therefore proposed the 3Vs principle for assessing the scientific validity of animal research. Moreover, the 3Vs together with the 3Rs and the HBA represent the principle of proportionality by which funders, regulators, and editors can formally assess the indispensability of animal research. Thus, to be deemed indispensable, a study needs to be suitable (determined by the 3Vs), necessary (determined by the 3Rs), and reasonable (determined by an HBA). Finally, as important societal interests determine the legitimate aims of animal research, we should treat results from animal research as common (public) goods. This further implies that animal research must be transparent, collaborative and efficient, the very principles of Open Research. Therefore, for animal research to be ethically responsible, it should also be based on preregistration of study protocols, unconditional data sharing, and comprehensive reporting of all results.

Presentation: Oral

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The future of validation: It's not all about ring trials

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The principles of validation in a regulatory context were first established in the 1990s and gained international recognition with the adoption of OECD Guidance Document No. 34 (GD 34) in 2005, where validation is defined as the process by which the reliability and relevance of a particular approach or method is established for a defined purpose. Even if these principles of validation remain relevant today and the process described in GD 34 was successful in pioneering the regulatory acceptance of non-animal methods, an evolution of practices is needed to embrace emerging technologies, the increasing complexity of the information measured (e.g., 'omics), and the need for data integration to address complex endpoints. Recognising this need, the OECD organised a workshop in 2022 to discuss how to prepare the Test Guidelines Programme for emerging technologies. Also, the European Commission submitted a proposal to the OECD to revise GD34 in order to address some key provisions. For example, the purpose and value of inter-laboratory ring trials within the validation process merits its discussion, considering how lengthy and expensive they can be. As more validation studies are sponsored and managed by method developers, ensuring data integrity, transparency and independent review of the study is becoming paramount as well. This talk provides an overview of the challenges and opportunities for adapting validation practices to keep pace with scientific progress whilst ensuring scientific confidence and the protection of human and environmental health. Examples of current validation studies applying new practices will be presented and discussed.

Presentation: Oral

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The process and importance of integrating NAMs in GHS

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Historically, hazard categories defined under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) have relied on bounds set exclusively by results from animal tests. However, there has been recent effort to include in GHS new types of non-animal information for classifying certain hazards, for example, skin corrosion/irritation, serious eye damage/eye irritation and skin sensitisation. These types of data are being given higher weight under GHS than before and can in many cases now be used on their own to conclude that no classification is warranted. One of the main features of GHS is the principle of test method neutrality, which permits the use of validated methods/approaches other than OECD Guidelines for classification purposes. The use of non-guideline methods may be particularly useful to classify into categories not covered by current OECD Guidelines. Another important evolution in GHS brought by the recent revision of the chapters on serious eye damage/eye irritation and skin sensitisation is the introduction of the concept of Defined Approaches and their use for classification. Importantly, for all endpoints mentioned above, the New Approach Methodologies (NAMs) incorporated were validated against the animal endpoints; therefore, classification using NAMs is straightforward. For other hazard categories, comparison with the animal endpoints may not be appropriate, and more innovative approaches may be needed to articulate categories that are amenable to description using NAM-derived data. This presentation will describe the GHS review process and lessons learned from recent experience that is relevant to future application of NAM data to classification.

Presentation: Oral

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In vitro determination of a point of departure for next generation risk assessment (NGRA) of skin sensitizers: Reproducibility and precision of the GARDskin dose-response assay

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New Approach Methods (NAMs) for the assessment of skin sensitizers have been adopted as OECD Test Guidelines (TGs), supporting hazard- and GHS potency classifications. However, more granular potency data are needed to derive a point-of-departure (PoD) for quantitative risk assessment (QRA) for identified skin sensitizers.

The GARDskin assay (OECD TG 442E) provides binary hazard classifications. A modified version of the protocol incorporating dose-response measurements has recently been described which uses linear models for the prediction of LLNA EC3/Human No Expected Sensitization Induction Levels (NESIL) values.

This study aims to perform a pre-validation exercise to evaluate precision and reproducibility of the assay and illustrate how it can be implemented into available NGRA-framework to determine safe use levels in consumer products by evaluating 17 fragrance materials in a blinded study. Reproducibility was assessed by evaluating 11 of the materials in three replicate experiments. Results illustrate that predicted LLNA EC3/human NESIL values correlate well with reference data (geometric mean fold-error: 3.8 and 2.5, respectively), and that the continuous potency predictions are reproducible between experiments (geometric mean fold-change: 2.9).

A case study using isocyclocitral was used to illustrate how the assay can be implemented into an NGRA-framework. The predicted NESIL value was used within a weight-of-evidence approach to derive a PoD for use in a QRA. Here, we were able to demonstrate how the method can be used as a source of information to derive a PoD and predict an acceptable exposure level to ensure product safety, avoiding the generation of new animal data.

Presentation: Oral

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Policy for science: The role of policymakers in supporting innovative science

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This presentation aims to present a methodology to map the evolution of European Parliament's positions on animal testing and alternative methods over time, across political groups, member states and national delegations in order to understand how disruptive innovations such as NAMs (New Approach Methodologies) can eventually be integrated into policy. The study relies on analysis of public votes of the Members of the European Parliament (MEP) related to animal testing and alternatives methods, using the database of Votewatch, an ex-Brussels-based NGOs collecting data on the reports put to vote in plenary. By analyzing the amendments' scope (ethical, reliability of the method, environmental or economic…) across reports and political mandates, and the votes they gathered across the political spectrum, the methodology intends to identify how new arguments and wording regarding NAMs and laboratory animals are perceived by MEPs throughout time. Ultimately the study will highlight how from an ethical concern, animal replacement is becoming a scientific issue or not, but also identify areas of concerns for policy makers explaining reluctancy to change. Ultimately, this study will help understand how to better communicate science to policy makers to improve legal acceptance of disruptive and promising technologies such as NAMs as well as providing a framework to perform this analysis with other topics.

Presentation: Oral

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Generation of an alveolus-on-chip model for personalized drug screening against viral-bacterial co-infections in viral pneumonia

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Secondary bacterial infections are a frequent complication of influenza virus (IV) infections that can manifest as seasonal epidemics and occasional pandemics. To date, there are no specific medications for treating IV – bacterial super-infections. Animal models and conventional cell culture approaches have been frequently used to study the underlying pathogenesis of co-infections. However, existing *in vitro* techniques typically fall short of accurately capturing the complexity of the lung, while animal models frequently can't adequately represent the human lung. Thus, novel patient-specific test systems are urgently required to develop effective anti-infective treatments for secondary bacterial infections.

Existing lung-on-chip models mostly use immortalized cell lines or primary cells which are both associated with inter-individual variability and the risk of allogeneic responses in immunocompetent models due to mixing up cell material from different donors. To overcome this limitation, human-induced pluripotent stem cells (hiPSCs) were differentiated into alveolar type II cells (AT2s) and complemented with isogenic iPSC-derived endothelial cells and macrophages. Using tissue engineering, we recreated the alveolar barrier with an air-liquid interface in a microfluidically perfused biochip. We were able to successfully infect AT2s with IV and used immunofluorescence microscopy to track cell type-specific progression of the infection process. By employing hiPSCs from various donors, we will evaluate novel medication options and the patient-specific response to viral-bacterial co-infections in the novel alveolus-on-chip model.

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[2] Deinhardt-Emmer, S. et al. (2020). *Biofabrication 12*.
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Presentation: Oral

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Data-driven decision-making using advanced high-throughput environmental risk assessment of fragrance materials

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For more than 20 years, environmental risk screening frameworks such as Salvito et al. [1] have been effective tools to evaluate the environmental safety of fragrance materials and reduce the need for bench-top toxicity testing. In light of the rapid growth in global use of fragrance materials, we propose an update to the Salvito et al. framework to expand its geographic scope and to incorporate recent advances in environmental exposure science and ecological hazard characterization. In this presentation, we describe the updated framework detailing its expanded geographies, real-time access to the most current population and hydrological data, estimated environmental exposure using wastewater treatment plant simulation models and biodegradation, material categorization based on mode of action (MoA), application of an MoA-based ecological threshold of concern, and its streamlined execution. The tool's methods and outcomes are illustrated through a proof-of-concept exercise. The updated framework is an enhanced risk assessment tool that enables users and suppliers of fragrance materials to perform timely assessments of thousands of fragrance materials in order to maintain a high degree of environmental protection and support science-based decisions related to product formulations.

Reference

[1] Salvito et al. (2002). *Environ Toxicol Chem 21*, 1301-1308.

Presentation: Oral

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Changing handling practices to improve animal welfare and scientific outcomes

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Refinements in everyday activities, particularly handling and restraint, are accessible to all that work with animals and have direct positive physiological impacts, particularly for mice, reducing chronic stress and improving scientific outcomes. Howev-

er, changing longstanding procedures is a challenge that requires a multi-pronged approach to build support for implementation of low stress handling (LSH) throughout an organization. This effort has required consensus building with management, operations, veterinarians, trainers, and those working in the rooms. A benchmarking survey of current practices across sites and business units globally was conducted to determine the prevalence of handling practices for infant/juvenile, young adults, pregnant, and older/less handled mice, and rats. Pilot studies to investigate the feasibility of LSH of mice in a production setting, tunnel handling specifically, were conducted within an isolator and a barrier facility. We also conducted studies at multiple North American Safety Assessment sites to identify preferred handling practices for rats. There have been multiple presentations to management, operation managers, trainers, discussions during site visits, as well as during role specific calls with behavior management, animal welfare, and resiliency building networks. E-learning materials and webinars have been provided to raise awareness of the benefits of LSH. These efforts have built consensus and support for implementation of LSH for mice and rats across multiple business units, at 79 sites, in 11 countries. Participants will learn from the many challenges encountered throughout this process and the valuable lessons learned that can inform others' efforts to implement LSH in mice and rats.

Presentation: Oral

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ASPIS strategy towards animal-free safety assessment of chemicals

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The safety of hundreds of thousands of chemicals in market products remains unknown due to the high cost and slow pace of traditional animal testing. To address this problem, the EU Research & Innovation Programmes invested €60 million to better understand the risks associated with chemical exposure by funding three consortia; RISK-HUNT3R, ONTOX, and PrecisionTox. These consortia are assembled into ASPIS (Animal-free Safety assessment of chemicals Project cluster for Implementation of novel Strategies), which currently consists of more than 70 institutions across 16 European countries and the U.S. ASPIS is adopting new approach methodologies (NAMs) using multi-species and *in vitro* omics technologies; robust *in vitro* and *in silico* methodologies; and artificial intelligence. This innovative science for societal change supports biomarker, quantitative AOP and database development. Furthermore, it is integrating these efforts to develop a Next Generation Risk Assessment Framework (NGRA) entitled ASPA (ASPIS Safety Profiling Algorithm). ASPA is a tiered approach that defines a decision logic to prioritize and filter information in order to guide data generation and interpretation for transparent and consistent decision-making. Ultimately, ASPIS will accelerate and improve animal-free chemical risk assessment in the EU through the development of NAMs and an NGRA framework to reliably detect, regulate, and mitigate human exposure to toxic substances. This knowledge allows for informed decisions in chemical risk assessment and regulatory acceptance that safeguard human and environmental health while facilitating the development of safe and sustainable products.

Presentation: Oral

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Different types of meta-analysis to replace animal experiments using literature data

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Background: Systematic reviews and meta-analyses can be used to find and select between new alternative methods, but if an adequate amount of literature is available, they can even be used to effectively replace new animal experiments. Implementation may increase with improved familiarity with these methods.

Aim: This presentation will describe new and known meta-analysis methods which can aid reduction and refinement of animal use throughout the field of laboratory animal experimentation, in a manner that is accessible and comprehensible also for attending scientists with relatively little statistical background.

Content: The discussed types of meta-analysis to replace animals by combining data from literature will at least comprise: 1.) simple meta-analyses which can be used instead of pilot experiments; 2.) meta-regression, which allows for interpolation and thereby predicting untested changes in experimental set-up; 3.) network meta-analyses, which allow for indirect comparisons of interventions; and 4.) cumulative meta-analyses, showing if and when overall research findings result in an overall stable outcome estimate, indicating that further experiments are no longer necessary. The theoretical explanations will be illustrated with examples from the laboratory animal sciences, providing examples of replacement and reduction of animal use.

Conclusion: The presentation will provide sufficient information on how to gather literature data reliably and reproducibly, and tips on how to get started with these types of analyses.

Presentation: Oral

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Recent developments in preclinical systematic review methodology

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Background: While systematic reviews are extremely useful in reducing and replacing animal research, they are currently not being used to their full potential. Reasons for this are: 1.) limited familiarity with the method, 2.) they require a lot of time, and 3.) the term "systematic review" is devaluating because of inappropriate use.

Aim: This presentation will aid to tackle all these reasons, by summarizing several important methodological developments for different parts of the review process.

Content: We will describe advances from our and other groups for all phases of the review process, starting with the selection of the appropriate review type. The use and limits of search filters will be briefly discussed, followed by the importance and problems with reporting search strategies in a reproducible manner. Next, different tools that can be used for screening and data extraction will be compared both qualitatively (focusing on user experiences) and systematically (with a feature analysis). Then we will highlight possible concerns with the implementation of AI using algorithms that have only been tested on human literature for reviews of animal studies and alternative methods, and our tests to assess if these concerns are legit. Furthermore, optimal processes for data extraction, and consistency between extractors, will be presented. The presentation will finish with and an evaluation of different methods for assessing study quality.

Conclusion: Systematic reviews remain time-consuming, but recent methodological developments can greatly aid reviewers.

Presentation: Oral

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Housing matters – Realistic human model systems depend on realistic exposure to pathogens

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The purpose of our analyses was to evaluate the impact of conventional compared to individually ventilated cages (IVC) housing for rodents. While the importance of the immune system on healing process and outcome was identified, mice housing became more hygienic and developed ever more towards highly sterile and sealed off conditions. Here we show a difference in bone healing outcome depending on different housing conditions with a significantly better healing under sterile conditions.

We investigated bone fracture models in mice and compared one group kept in conventional housing conditions to another kept in IVCs. Surgery, surgeon, post-surgery lifetime as well as all treatments were identical. The housing implied significant differences in the immune composition of the animal groups. A significantly higher percentage of effector memory T-cells in the conventionally housed animals caused a significant delay in bone healing. A therapeutic approach to intentionally treat delayed bone healing only showed promising results in IVC housed animals with a naïve immune composition. In conventionally housed animals, however, this same therapeutic approach significantly worsened bone regeneration in 50% of the tested animals, due to elevated effector memory T-cell subsets in those animals.

We conclude that housing in IVCs does not allow the maturation of an immune experience and – in addition – impacts the healing outcome. Thus, any results found and conclusions drawn from IVC housed animals should be carefully reconsidered. Especially in a translational setting such housing conditions may not allow to properly predict any patient-relevant pre-clinical evaluation of therapeutic concepts.

Presentation: Oral

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Automated platform for creating patient-derived glioblastoma organoids and high throughput drug screening

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Glioblastoma (GBM) is the most prevalent primary intrinsic brain tumor and amongst most lethal forms of cancer with a median survival of 14.6 months. Current drug development efforts are hindered by inadequate models that does not recapitulate the tumor's complexity. Recent studies suggest the presence of self-renewing, tumor-propagating cancer stem cells (CSCs) play a crucial role in the resistance to conventional therapies. We developed a method to use CSCs isolated from GBM patients to creating vascularized multi-zonal patient derived GBM microtumors suitable for drug screening. The microtumors display multiple tumoral niches, a vast cellular heterogeneity, clinical features and treatment re-

sponses. Furthermore, we developed a method for precise robotic deposition of these microtumors, allowing micro-sensors embedding, real-time metabolic monitoring and dye-free high throughput drug screening, with performance surpassing traditional *in-vivo* models. Kinetic screening of 126 FDA approved anti-cancer drugs in patient-derived organoids with different backgrounds uncover unique universal response patterns to different cancer treatments. Concurrent screening conducted on liver organoids revealed that slow-acting drugs, which are commonly missed in standard screens due to their delayed effect, seem to damage CSCs particularly, while exhibiting reduced toxicity in neighboring tissues. The screen evaluates drugs based on their therapeutic window, rather than cytotoxicity, detecting more effective treatments. Network analysis uncovers novel pathways that shed light on basic mechanisms underlying the CSCs response to anti-cancer treatment. Our platform offers a new approach for drug development and screening for complex tumors, providing valuable information for both novel drugs and repurposing attempts.

Presentation: Oral

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Efficacy and safety of metabolic interventions for the treatment of severe COVID-19: *In vitro,* observational, and non-randomized open label interventional study

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Viral infection is associated with a significant rewire of the host metabolic pathways, presenting attractive metabolic targets for intervention. We chart the metabolic response of lung epithelial cells to SARS-CoV-2 infection in primary cultures and COVID-19 patient samples and perform *in vitro* metabolism-focused drug screen. We perform observational analysis of Israeli patients hospitalized due to COVID-19 and comparative epidemiological analysis from cohorts in Italy and the United States. In addition, we perform a prospective non-randomized interventional open-label study in which 15 patients hospitalized with severe COVID-19 were given 145 mg/day of nanocrystallized fenofibrate added to the standard of care.

SARS-CoV-2 infection produced transcriptional changes associated with increased glycolysis and lipid accumulation. Metabolism-focused drug screen showed that fenofibrate reversed lipid accumulation and blocked SARS-CoV-2 replication through a PPARa-dependent mechanism. Analysis of 3,233 Israeli patients hospitalized due to COVID-19 supported *in vitro* findings. Patients taking fibrates showed significantly lower markers of immunoinflammation and faster recovery. Additional corroboration was received by comparative epidemiological analysis from cohorts in Europe and the United States. A subsequent prospective non-randomized interventional open-label study was carried out on 15 patients hospitalized with severe COVID-19. Patients receiving fenofibrate demonstrated a rapid reduction in inflammation and a significantly faster recovery compared to patients admitted during the same period.

Taken together, our data suggest that pharmacological modulation of PPARa should be strongly considered as a potential therapeutic approach for SARS-CoV-2 infection and emphasizes the need to complete the study of fenofibrate in large randomized controlled clinical trials.

Presentation: Oral

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Roadmap for NAMs-mixtures assessment: Environmental pollutants

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The assessment of environmental pollutants a) involves thousands of chemicals; b) requires aggregation of oral and inhalation exposures; and c) each individual has different exposure patterns. This has triggered conceptual approaches, such as "individualized exposomes"; supported by geographically rereferred models. Technological developments are offering innovative tools for conducting environmental health assessments, getting adequate toxicity data is the main limitation. NAMs appear as the ideal solution but requires new conceptual approaches. This session will present a roadmap currently under development as part of the Horizon Europe Partnership PARC https://www.eu-parc.eu/. The scientific hypothesis supporting this proposal combines NAM-based mechanistic understanding of mixture toxicity, with *in silico* modelling tools for aggregating exposure and effects. The roadmap integrates ecological status and human health through landscape risk assessments under "One Health". The approach supports sustainability assessments for combined exposures and also the use of ecological systems as environmental health sentinels. Generic landscape scenarios cover the urban environment; complemented with local point-sources for industrial emissions and hot-spots, while landscape agricultural scenarios cover pesticides and other agrochemicals. Exposure via food and drinking water is addressed at "population group" level, combining diets with environmental parameters, monitoring/market data, and landscape-based predictions.

In vitro approaches and AOP networks are proposed for the integration of combined effects. The novelty is to quantify the progression towards adversity through the Key Events Relationships (KER), as combined exposure modulates the KER, also for chemicals acting through other AOPs indirectly connected as part of a network. Case studies will be presented for discussion.

Presentation: Oral

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Chemical mixture risk assessment in Europe

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Data driven approaches for risk assessment of combined exposure to multiple chemicals are successfully applied by regulators to ensure a high level of food safety. For pesticide active substances cumulative assessment groups (CAGs) were built based on comprehensive toxicity data. Cumulative risk assessment (CRA) was performed for these CAGs considering wide-ranging exposure information also for sensitive parts of the population based on conservative assumptions. So far, no elevated risk has been detected for the CAGs on thyroid and nervous system due to combined exposure to multiple pesticide residues.

For other groups of compounds with less comprehensive data packages like contaminants, structure based grouping and relative potency factors are used for CRA. Additionally whole mixture approaches, taking into account exposome data and new approach methods (NAM) for mechanism and adverse outcome pathway driven hazard identification are discussed. Their implementation would facilitate CRA across regulatory domains.

A roadmap for action on risk assessment of combined exposure to multiple chemicals (RACEMiC) was prepared. Within a number of European projects such as EuroMix or more recently PARC steps toward the development and implementation of such methods have been undertaken.

Presentation: Oral

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Formal courses for college students to sensitize about NAMs: Catch them young

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"An obstacle in shifting the current research paradigm is the limited availability of educational and training courses on animal free methods and approaches" (Hermann et al., 2019). There must be effort to inculcate the ideas and practice "when young" so that when these clientele take to science as career they are already sensitized about and infused with confidence for practice the NAMs. This was realized in India in the context of establishment of Mahatma Gandhi-Doerenkamp Center (MGDC) by the Doerenkamp-Zbinden Foundation, Switzerland, which later became the National Center for Alternatives to Animal Experiments (NCAAE), and then the Society for Alternatives to Animal Experiments. The Centers and the Society have been engaged in training college and university students in *in vitro* and *in silico* approaches, and alternative model organisms. Starting 2010, more than 30 such training workshops, with clearly defined curricula, have been conducted. Industry and CRO partners facilitated the transformation. A four-credit elective course has been designed and offered to the graduate students of different streams of Life Sciences. Special effort, with support from voluntary organizations, was put to discontinue dissections as aspect of learning of human and animal anatomy from school and college curricula, and digital alternatives have been introduced. The more than 100 training workshops to college and school students, and persistent pressure on the regulatory authorities made this possible. These endeavors may be adopted elsewhere as well to bring up the next and upcoming generations of students and trainers adequately sensitized, informed and trained in NAMs.

Presentation: Oral

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History in the making: The European Citizens' Initiative to End Animal Testing

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In 2021, animal protection organisations united across Europe, with the support of key industry stakeholders, to launch the "Save Cruelty Free Cosmetics – Commit to a Europe Without Animal Testing" European citizens' initiative (ECI). Addressing key policy threats to and opportunities for advancing animal-free science, the initiative called for robust implementation of the existing EU ban on animal testing for cosmetics ingredients, for a transformation of chemicals regulation by focusing on the implementation of non-animal methods, and for a commitment to plotting a phase-out of all experiments on animals. As a formal mechanism by which European nationals can call on the European Commission to propose new legislation, an ECI must gain the support of at least 1 million EU citizens in the form of signatures, reaching a minimum threshold across at least seven different countries. Following a provocative year-long campaign, this initiative was validated with over 1.2 million statements of support and passed the minimum target in an outstanding 22 different countries, demonstrating pan-European support for ending animal testing. At the time of writing, organisers of the initiative are due to meet with Commission representatives and to be offered a hearing in the European Parliament before a final decision is expected on the legislative proposals late in July 2023. Marking a pivotal moment in history, we summarise the trajectory of the initiative, make recommendations to support the transition to animal-free science, and draw conclusions on the outcomes and next steps of the bold policy proposals made.

Presentation: Oral

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Pain in experimental animal studies and the non-affiliated member?

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Any study that is likely to cause "severe" suffering in experimental animals requires serious review in an appropriate way. Each nation or jurisdiction has established minimum methods and criteria for this effort. In the United States, we operate under guidelines from the national Public Health Service and the US Dept of Agriculture. We also have to deal with the New York State Health Department. As the non-affiliated member of our Institutional Animal Care and Use Committee (IACUC) for the past 10 years, I have been privy to many protocols dealing with these issues, including Category E protocols, and the serious, often lengthy discussions in our committee. Some of the issues we have dealt with are: post-operative pain management, criteria for humane endpoint determinations and adequate monitoring of animals having undergone extensive surgery. I will share how the IACUC reviews proposals, mitigates pain and suffering of subject animals and, most important to me, the essential contribution of the non-affiliated member to the entire process.

Presentation: Oral

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The next evolution in animal welfare assessment standards

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Purpose: The Canadian Council on Animal Care (CCAC) is the national peer-review organization responsible for setting, maintaining, and overseeing the implementation of standards of ethical care and use of animals in science throughout Canada. In 2021 the CCAC published new national standards for animal welfare assessment, and in 2023 is set to publish new standards on categories of welfare impact.

Process: The standards update process included the synthesis of evidence and expert opinion by an expert subcommittee with both an external expert and public review of drafts. In addition, the process includes oversight from representatives of national members organizations.

Changes: Together, these standards shift the focus away from the scientific procedures performed on animals and towards understanding how the animals experience the world in which they

live, that is, their welfare. Going beyond daily health checks, welfare assessments should be an integration of information collected during husbandry activities, during and after experimental procedures, and any additional assessment tools required. It is crucial to recognize that an animal's welfare is impacted by more than just the scientific procedures performed on it; their environment and phenotype can also have large impacts on their welfare.

Conclusion: These new national standards shift away from focusing on animal health and the procedures performed on animals. Instead, these national standards are promoting a holistic focus on the experiences of individual animals. This is an important shift in the culture of animal use and is the next step to improving scientific animal welfare in Canada.

Presentation: Oral

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Rethinking endpoints – Setting national standards for the 21st century

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Purpose: The Canadian Council on Animal Care is the national peer-review organization responsible for setting, maintaining, and overseeing the implementation of standards of ethical care and use of animals in science throughout Canada. In 2022 the Canadian Council on Animal Care published updated national standards for the identification of scientific endpoints, humane intervention points, and cumulative endpoints.

Process: The standards update process included the synthesis of evidence and expert opinion by an expert subcommittee with both an external expert and public review of drafts. In addition, the process includes oversight from representatives of national members organizations.

Changes: The standards introduce the concept of "humane intervention points". When the criteria for humane intervention points are met, an intervention to address the negative welfare state is required, but this action is not necessarily euthanasia. Welfare-protecting interventions must occur as early as possible and can range from supportive care to removing animals from scientific activity. In addition, the new standards set requirements for cumulative endpoints: the point at which an individual animal should be considered to have reached their lifetime maximum involvement in scientific activities. Importantly, animals should be subjected to only one severe or high welfare impact experience in their lifetime (Category D or E) and the assessment should incorporate both physical and psychological impacts.

Conclusion: The new national standards require earlier interventions to reduce negative welfare impacts and set a clear limit on severe/high welfare impact experiences for animals used for research, testing, and teaching in Canada.

Presentation: Oral

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Technical framework for enabling high quality measurements in new approach methodologies (NAMs)

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New approach methodologies (NAMs) are *in vitro*, *in chemico*, and *in silico* or computational approaches that can potentially be used to reduce animal testing. For NAMs that require laboratory experiments, it is critical that they provide consistent and reliable results. While guidance has been provided on improving the reproducibility of NAMs that require laboratory experiments, there is not yet an overarching technical framework that details how to add measurement quality features into a protocol. In this manuscript, we discuss such a framework and provide a step-by step process describing how to refine a protocol using basic quality tools: cause-and-effect analysis, flowcharts, check sheets, control charts, histograms, and scatterplots. The steps in this framework include 1) conceptual analysis of sources of technical variability in the assay, 2) within laboratory evaluation of assay performance, 3) statistical data analysis, and 4) determination of method transferability (if needed). While each of these steps has discrete components, they are all inter-related and insights from any step can influence the others. Following the steps in this framework can help reveal the advantages and limitations of different choices during the design of an assay such as which in-process control measurements to include and how many replicates to use for each control measurement and for each test substance. Overall, the use of this technical framework can support optimizing NAM reproducibility, thereby supporting meeting research and regulatory needs.

Presentation: Oral

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Technical evaluation of an oral irritation assay using 3D constructs

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Biocompatibility testing using *in vivo* tests is often one of the final evaluations of new dental materials. To reduce the likelihood of failure at this late stage and reduce *in vivo* testing, predictive biocompatibility testing using new approach methodologies (NAMs) such as *in vitro* methods is needed. In this study, we describe a technical evaluation of an oral irritation test by evaluating changes in the viability of 3D constructs using the MTT assay, with Epi-Oral constructs as a case study. These 3D constructs more closely resemble the human oral environment than 2D models based on a single cell type. We first analyzed the assay using conceptual tools such as cause-and-effect analysis and flow charts to design an initial plate layout. Then, we performed comprehensive evaluation of the sources of uncertainty in the assay by investigating pipetting, the control measurements to include, and the repeatability of key control measurements such as the negative, solvent (sesame seed oil and saline solution), and positive controls (1% Triton-X). We also tested several test compounds such as the Y-4 polymer, with a known irritant added, and dentally relevant substances such as sodium dodecyl sulfate (SDS) at varying concentrations. Lastly, a statistical model was built to support assay design and determination of evaluation of test substances as yielding a positive or negative result. By varying the number of replicates and the α values, different limits of detection and likelihood of false positive and false negative results could be specified.

Presentation: Oral

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Caring for our people caring for our animals: Promoting compassion fatigue resiliency

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Working with research animals can be both rewarding and challenging. Workplace stress can be exacerbated due to scientific requirements for animal models with stress/pain, end of study euthanasia, and societal stigmatization about their job. Some institutions try to proactively prepare individuals for these situations with internal compassion fatigue resiliency programs. However, currently these programs are neither widespread nor formally and openly evaluated. Therefore, the purpose of this project was to develop institutional resources and evaluate them via a longitudinal intervention trial.

Six research institutions were recruited to participate in a two-year pre-post intervention trial. A mixed methods survey was developed to evaluate professional quality of life, stress, resiliency, job satisfaction, retention, and factors influencing compassion fatigue resiliency. Quantitative data were analyzed via general linear models and qualitative data were analyzed by theme.

Baseline data was collected from 200 participants. Personnel who reported higher compassion satisfaction also reported higher retention and job satisfaction. Conversely, personnel who reported higher burnout also reported lower job satisfaction. In response to open-ended questions, participants said their compassion fatigue was impacted by institutional culture (70% of participants), animal research (58%), general mental health (41%), and specific compassion fatigue support (24%).

In conclusion, these results show that professional quality of life is related to important operational metrics of job satisfaction and retention. Furthermore, compassion fatigue is impacted by factors beyond working with research animals, including institutional culture and general mental health support. Overall, this project provides rationale, resources, and insight for institutional support of compassion fatigue resiliency.

Presentation: Oral

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A six-step strategy for a roadmap towards animal free-science in the United Kingdom

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The UK government has stated its ambition for the nation to become a "science superpower" by 2030. However, a recent parliamentary report highlighted how the country's science and technology strategy is "unfocused" and lacks an overarching vision and implementation plan, meaning there is a risk that this goal could become an empty slogan. The UK needs a government-led strategy for basic research and regulatory testing to position itself at the forefront of global science. Building on international progress in transitioning to animal-free science, a six-step strategy has been developed that can support the UK government in accelerating the UK's transition. One of the key objectives of this strategy is developing a roadmap to phase out the use of animals in research, testing, education and training, and to accelerate the uptake of ad-

vanced technologies that outperform animal-based methods. UK industry reports have highlighted concerns over the translation of research on animals to human clinical benefits, as well as identifying the potential business opportunities arising from accelerating the uptake and development of superior human-relevant non-animal approaches, including the potential to drive economic growth and attract international investment. This six-step strategy provides concrete actions that can feed into a government-led vision and implementation plan to equip the UK to take a lead in the worldwide paradigm shift in biomedical research and testing. This presentation will outline the progress the UK is making towards such goals and some of the challenges that still need to be overcome.

Presentation: Oral

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A liver and testis multi-organ-chip: Towards a systemic male reprotoxicity model

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Current benchtop reprotoxicity models typically do not include interactions of the liver-testis axis. However, these are important to study the biotransformation of substances.

Here, Testicular organoids and liver spheroids were co-cultured in a multi-organ-chip circuit for a week. Additional single-organ-chips and well plates (static) were loaded only with testicular organoids or liver spheroids for comparison. Subsequently, the system was challenged with cyclophosphamide, a prodrug that has demonstrated germ cell toxicity after its bioactivation in the liver, to replicate the systemic human liver-testis interaction *in vitro*. Single-chip testicular organoids were used as a control.

The Co-culture experiment revealed that the specific medium was able to maintain the metabolic activity of the tissues. Additionally, the testicular organoids developed optimally and generated higher inhibin B values, though the testosterone levels were not as high as in the static culture with the testicular organoid-specific medium. By comparison, testosterone secretion by testicular organoids cultured individually on multi-organ-chips reached a similar level as the static culture at Day 7. This suggests that the liver spheroids have metabolised the steroids in the co-cultures, a naturally occurring phenomenon. The addition of cyclophosphamide led to upregulation of specific cytochromes in liver spheroids and loss of germ cells in testicular organoids in the multi-organ-chip but not in single-testis-culture.

This co-culture model responds to the request of a specific tool that enables the testing of candidate reprotoxic substances with human biotransformation. It further allows the inclusion of other human tissue equivalents for chemical risk assessment on the systemic level.

Presentation: Oral

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Development of composite measure schemes for evidence-based severity assessment and refinement in neuroscientific research

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The multidimensional character of distress and pain complicates reliable assessment of suffering in laboratory animals. Therefore, composite measure schemes combining various parameters are usually required to obtain robust information about an animal's distress. In this project, we aimed to identify sensitive parameters for a combination in composite measure schemes as a basis for comparative severity assessment.

Comprehensive data sets comprising behavioral and biochemical data were obtained from induced and genetic mouse models of neurological and psychiatric disorders. Pre-selection of parameters was based on earlier findings, including an assessment of the development course in young C57BL/6J mice. The data were subjected to a bioinformatics workflow.

In young mice, age-dependency was demonstrated for different parameters, including saccharin preference, burrowing, wheel running, open field activity, and fecal corticosterone metabolites. Correlation and principal component analysis resulted in a condensation of information for the design of composite measure schemes. Data were then subjected to cluster analysis as a basis for severity classification. The classification system allowed the allocation of individual animals to different severity levels and a direct comparison of animal groups and models.

The findings indicate that the developmental course in young mice must be considered when developing multidimensional severity assessment schemes. The bioinformatics approach con-

firmed the suitability of the composite measure schemes for evidence-based severity assessment in adult and young mice. The schemes can be used for individual severity classification, comparisons between experimental groups or different models, and validation of refinement measures.

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Presentation: Oral

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An integrated research and testing strategy to go beyond the 3Rs

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Our company has developed an innovative approach, The Integrated Research and Testing Strategy (IRTS), which backs the principles of the 3Rs, in line with regulations, international standards, and our own Corporate Policy on the Protection of Animals.

Our company-wide strategy (IRTS) supports our goal of reducing reliance on animal models. It reinforces rigorous, state-of-the-art science as our North Star for selecting the best available, feasible, and translatable models to address scientific questions and adhere to regulatory requirements.

We take proactive measures to reduce the need for animals in research and testing by:

- Ensuring our selection of models is based on sound science,
- Fostering breakthrough innovation for consistent, robust translational research and testing,
- Facilitating regulatory acceptance of novel technologies and models, and
- Educating, training, and communicating with our colleagues and partners to embed IRTS across Sanofi sites and those of our affiliates.

By 2030, we aim to reduce the number of animals used in research and testing by 50%, compared to 2020. To achieve our 50% reduction target, we will:

- Phase in New Approach Methodologies,
- Challenge and waive obsolete animal tests,
- Replace animal models through validation, qualification, and acceptance by the competent authorities,
- Improve animal use as set out in our preclinical package study rationales and designs.

Presentation: Oral

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Experience with gravimetric testing in the DPRA data: Comparison to *in vivo* reference data

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Chemical reactivity towards skin proteins has been described as the molecular initiating event (MIE) of the adverse outcome pathway for skin sensitization. The direct peptide reactivity assay (DPRA) has been adopted as OECD test guideline 442C. In its currently adopted version, negative results (i.e., indicating a test substance is not protein reactive) are only acceptable if the test substance is a mono-constituent, organic chemical – and a molar test concentration of 100 mM is not producing peptide depletion in the DPRA. While technically it is feasible to assess test substances of unknown composition using a mass instead of a molar concentration (gravimetric procedure), no such protocol is currently adopted.

In the present study, we complied DPRA and *in vivo* skin sensitization data of 7 silicone materials, 33 agrochemical formulations,14 plant extracts, 15 polymer materials and 17 other test material with undefined molecular weight. The results of the DPRA were compared to the results of local lymph node assays and/or guinea pig skin sensitization test.

While either sensitivity, specificity or both were low for silicone materials, plant extracts, polymer materials and agrochemical formulations, the 17 other test materials with undefined molecular weight correlated with the *in vivo* results (78% sensitivity, 75% specificity and 76% accuracy). More substances representing the different substance classes should be tested to define where the gravimetric DPRA is applicable with good predictivity.

The broadening of the applicability domain of OECD 442C to substances without defined molecular weight would avoid otherwise necessary animal testing.

Presentation: Oral

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A human integrated organ system (MPS) for developing pharmacokinetic and organ toxicity data *in vitro*

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The development of an integrated organ MPS system that can be used to identify pharmacokinetic parameters and organ toxicity is a key first step in developing predictive (MPS) models. The Aim of this study was to study Acetaminophen and Cycloheximide kinetics and toxicity in a three-organ system. The complete test circuit consisted of human intestine, primary hepatocytes, and kidney cells (HK-2). Organ compartments were linked via a simulated blood system. Communication between organs occurred via osmotic flow across semi-permeable membranes. Simulated blood (PBS + 0.4% human serum albumin) flow was achieved with a micro syringe pump. Acetaminophen (APAP) (38 µg) and Cycloheximide (4.2 µg) were applied in a volume of 0.1 mL to the apical side of the intestinal chamber and then incubated at 37oC with 5% CO_2. Samples were collected from all organ compartments at multiple times (0.5, 1, 3, 6, 24, and 48 h). Cytotoxicity was monitored by LDH, MTT and with a panel of 12 genes associated with oxidative stress and inflammation. The resulting kinetic curves showed a peak absorption of 27.2 µg, with a time to maximum of 4.2 h for APAP and 4.5 µg peak, with a time to maximum of 5.5 h for Cycloheximide. APAP liver toxicity was low, but for Cycloheximide liver and renal toxicity were high. In conclusion, the MPS platform described here demonstrated that *in vitro* pharmacokinetic and toxicity data can be obtained and that these findings have relevance to *in vivo* data in the published literature.

Presentation: Oral

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Public awareness of human-relevant, animal-free science: Gaining support through effective communication

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The imperative to change to an animal-free research paradigm is widely recognized for two critical reasons. One is the enduring issue of ethics and animal suffering. The other is the societal need for better quality, biomedical science. Communicating this to public audiences remains both vital and challenging, in no small part because animal research continues to be shielded from scrutiny, maintaining a public impression of "secrecy".

Furthermore, when animal research attracts media attention, it is often accompanied by sanitized information or rhetoric. Two examples are the popular (and untrue) mantras that "animals are only used when necessary" or "animals can only be used when there is no alternative".

Public awareness of animal research is diverse. Many are surprised to find it continues (at a rate of > 190 million animals yearly, worldwide). Others are aware of animal tests for specific purposes (e.g., cosmetics, drugs). There is perceivably greater awareness of "animal testing" of products (toxicity testing), rather than "animal experiments", e.g., basic research; the latter being responsible for the vast majority of animal use and with no legal or regulatory requirement.

Similarly, awareness of animal-free New Approach Methods (NAMs) is comparatively low but gaining attention, corresponding with their increased scientific development over the last decade, driven by a need for better, clinically relevant science in drug and disease research, or chemical safety assessment.

This presentation reflects on public attitudes towards animal testing and ways to communicate on human-relevant research in the public's name, by using clear, concise and factual information.

Presentation: Oral

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The 3Rs collaborative: Creating evidence-based, practical, and impactful 3Rs change

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Creating widespread institutional change to advance the 3Rs is a challenging and important task. In the USA, where a particularly large number of research institutions are spread out across the country – making change can be especially difficult. The 3Rs Collaborative (3RC) is a non-profit whose mission is to promote better science – for both people and animals, by facilitating collaborative 3Rs opportunities.

The 3RC currently has 3 strategic pillars. To create a research landscape that is knowledgeable and supportive of the 3Rs. To facilitate targeted efforts towards specific, high-impact, evidence-based, and practical 3Rs techniques. And to drive general collaboration with and awareness of 3RC resources and programs.

For the first pillar, the 3RC is creating a 3Rs certification course and extensive resources for both individual and institutional support of compassion fatigue resiliency – with formal evaluation of

each. For the second pillar, the 3RC has identified 5 key focus areas: rodent health monitoring, refined mouse handling, translational digital biomarkers, microphysiological systems, and *in silico* technologies. For each focus area, the 3RC fosters collaboration and thought leadership between key stakeholders, works to understand current implementation and barriers, and then supports adoption by creating extensive practical resources.

In conclusion, the 3RC provides key strategic support to the USA and global research arenas. As a result of their efforts, at least 12 institutions have changed key practices in support of the 3Rs. Ultimately, the 3Rs Collaborative is a key player in the world of 3Rs centres.

Presentation: Oral

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Collaborative initiatives supporting reduction of NHP use in drug development

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The use of animals for safety testing within drug development programs is a balance between providing appropriate data allowing regulatory assessment of risk in humans, and applying the 3Rs principles (Replacement, Refinement and Reduction) for animal use.

Consortia efforts partnered with Regulatory Agencies have played a crucial role in shaping drug development best practices. Recent discussions have focused on promoting alignment and advancing science and best practices in key areas related to preclinical safety of oncology therapeutics, in light of the shortages of NHPs caused due to supply constraints and prioritized biomedical research.

For the development of therapeutics for oncology indications, opportunities were identified for the acceptance of abbreviated toxicology packages, without the compromise of a rigorous safety evaluation, utilizing a) CD3-bispecific antibodies, b) Antibody-Drug Conjugates (ADCs) with cytotoxic payloads and c) biotherapeutics for well-characterized targets such as PD-1 or VEGF. Results from consortia surveys and case-studies from industry sponsors will be presented on these topics where available.

There remains to be many opportunities for the continued streamlining of data packages (including innovative approaches for alternative technologies and animal study designs) for the selection of a safe First-In-Human dose, based on a totality of data resulting from an assessment of target biology, pharmacology and toxicology studies.

Presentation: Oral

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What key ethics principles should be: Setting national standards for the 21st century

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The Canadian Council on Animal Care's Ethics of Animal Investigation has served as guiding ethics principles in Canada for over thirty years. Since its publication, societal values, understanding of animal welfare and research methodologies have evolved, requiring a new Canadian standard fit for 21st Century science.

CCAC standards are developed by subcommittees, with members drawn from a wide range of relevant backgrounds. The Ethics subcommittee members (including researchers from a variety of disciplines, veterinarians, animal care personnel, animal welfare scientists and ethicists) developed CCAC Principles for the Ethical Use of Animals in Science which has undergone two levels of scrutiny: a peer review and a public review and will be published in the coming months.

The new document rests on Three Core Principles – Non-Maleficence; Sufficient Benefit; and Respect, thus aligning closely with principles for scientific activities involving humans. A further set of principles of application provide practical interpretation of the core principles when considering an animal-based based scientific activity. Although the 3Rs provide a strong ethical framework they do not ensure that animal-based activities are morally acceptable, hence the new principles incorporate and extend Russell and Burch's principles of Replacement, Reduction and Refinement.

These new principles set out the Canadian ethical framework for animal-based use in 21st Century science. The new principles of Non-Maleficence, Sufficient Benefit, and Respect, support an examination of scientific activities by all participants in the ethical use of animal in research, testing, and teaching.

Presentation: Oral

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Defenceless: Animal-based trauma training in the Canadian military

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Although the federal government is currently investing heavily in modernizing the Canadian military, its contributions fail to address the Department of National Defence (DND)'s trauma training program which provides instruction to the country's military medics. That training program, called "live tissue training," relies on the use of live animals – 10-week-old piglets – which even the DND recognizes make poor models for training medics to treat human injuries in the field. In fact, the DND admits that its use of pigs may even interfere with effective training by producing what it calls "training scars."

Animal Alliance of Canada and the Animal Protection Party of Canada reviewed over 3,200 pages received in response to Access to Information requests to the DND on its "live tissue training" program. Our presentation will provide detailed insight into our findings, including the heavy toll the training exacts on animals and the serious risk it poses to Canadian soldiers.

Seventy-seven percent of NATO member nations no longer use animals for military medical training. These countries have replaced animals with human patient simulators which accurately mimic human anatomy and physiology. Studies by the Canadian and U.S. militaries show this training to be as effective – or better – than training involving animals, as well as more cost effective. Our presentation will provide an overview of these alternatives as a way forward for the Canadian military.

Presentation: Oral

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Defined approaches for skin sensitization for diverse chemical sets

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While several non-animal methods have been accepted to identify potential skin sensitizers, none is considered a full replacement for animal tests. Defined approaches (DAs) combining multiple assays representing key events from the skin sensitization adverse outcome pathway have been accepted internationally under OECD Guideline 497 for hazard and potency prediction. This project evaluated 185 chemicals nominated by federal agencies with mandates for skin sensitization assessments: NTP, EPA, and the U.S. Consumer Product Safety Commission. We tested chemicals using three DAs that combine results from three assays: the direct peptide reactivity assay, the KeratinoSens™ assay, and the human cell line activation test. Local lymph node assay results were used as reference data. Individual test method accuracy for hazard (sensitizer vs. nonsensitizer) ranged from 40% to 80%. Accuracy for hazard classification using the 2 out of 3 DA was 46% to 89%, the Integrated Testing Strategy (ITS)v2 DA was 41% to 100%, and the Key Event 3/1 Sequential Testing Strategy (STS) DA was 31% to 100%. Accuracy was lower for classification of pesticide products, possibly due to heterogeneity and insolubility of some products. Potency prediction was based on GHS categories. Correct potency classification using the ITSv2 DA ranged from 37% to 53%, while the 3/1 KE STS DA ranged from 27% to 67%. DAs based on *in vitro* methods may provide useful alternatives to animal testing for predicting skin sensitization hazard and potency of substances relevant to multiple U.S. agency programs.

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Presentation: Oral

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ChemDIS-ZF: A computational chemical-disease inference system for zebrafish

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Zebrafish have recently emerged as an effective model organism for toxicological investigations and drug discovery. Their small size, high reproduction, and transparency make them ideal for identifying the biological effects of chemical exposure. However, it is still infeasible and unethical to apply zebrafish models for a large number of chemicals. Computational methods are potentially useful for prioritizing the use of zebrafish models. In this study, we present a chemical-disease/phenotype inference system for zebrafish (ChemDIS-ZF) that predicts the potential associa-

tions between chemicals and zebrafish diseases and phenotypes. ChemDIS-ZF integrated chemical-protein interaction data from the STITCH database with gene-phenotype and gene-disease interaction data from the Zebrafish Information Network (ZFIN). An enrichment analysis tool was developed to derive chemical-protein-disease/phenotype associations that could shed lights on the potential underlying mechanisms. Currently, 419,328 chemicals, 23,180 zebrafish proteins, 3,616 diseases, and 3,104 phenotypes for zebrafish were included in the system. ChemDIS-ZF is expected to reduce the use of zebrafish experiments and accelerate the chemical toxicity evaluation and drug discovery.

Presentation: Oral

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Incorporating *in silico* methods into genotoxicity risk assessment: Moving beyond mutagenicity

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Use of *in silico* predictions for genotoxicity is common practise across all chemical industries. Although prediction of bacterial mutagenicity is lauded and accepted in lieu of experimental data for regulatory assessment of impurities in pharmaceuticals under the ICH M7 guideline, the reliability of models for other genotoxicity endpoints (e.g., chromosome damage) and assays (e.g., chromosome aberration and micronucleus test) is not considered optimal. With the future of toxicology assessment moving towards a world less reliant on animal testing, the need to improve models for genotoxicity endpoints other than mutagenicity is paramount. For instance, two EFSA scientific panels, Plant Protection Products and their Residues and Food Additives and Flavourings, have both published scientific guidance requiring use of *in silico* predictions to assist genotoxicity assessment. A review of the status of *in silico* tools for the assessment of genotoxicity identified key factors that enable the uptake of mutagenicity for regulatory assessments but hinder the use of models for other endpoints. For each specific use case, it is important to understand how models fulfil the requirements. What data is available for modelling? How are the reported results to be used in a regulatory assessment? Does the data alone provide sufficient information? This knowledge enables model developers to engineer new solutions that leverage the power of existing and emerging technologies for challenging genotoxicity endpoints. Ultimately, development of new data sets and models for a broad set of genotoxicity endpoints will empower safety assessors to embrace a regulatory landscape which integrates *in silico* systems.

Presentation: Oral

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How can funders improve transparency and quality of animal research? A case study

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Via requirements, funders can have a large impact on how research is planned, performed, reported and shared, yet researchers rarely have their say on these policies. When it comes to animal studies, methods that can improve its transparency and quality have come increasingly into attention. However, it is important to understand the impact of these requirements for researchers and surrounding stakeholders to increase the effectiveness of policies, preventing unnecessary bureaucracy.

Hereby we present an evaluation of the Netherlands Organisation for Health Research and Development's funding policies. This qualitative case study aimed to assess the relevance of transparency methods (i.e., preregistration, FAIR data, ARRIVE guidelines and open access publishing) and identifies barriers and facilitators to compliance. Over a period of 2 years, input was gathered from grantees and other key stakeholders (e.g., animal welfare bodies, funders, data stewards, universities) through questionnaires and interviews. Data are processed using thematic analysis.

Preliminary results show that researchers see the value of the transparency requirements for themselves, their research and their peers. Moreover, further training, guidance and support (also financially) from both funding agencies and their institutes are mentioned as facilitators to better comply. Preregistration, in its current form, seems to be the most controversial of all requirements due to administrative burden and belief toward its complexity and lack of flexibility.

The results of this evaluation will be comprised in an advisory report and translated to concise recommendations to funders and other stakeholders on improving compliance and creating incentives for better animal research.

Presentation: Oral

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Long-read transcriptome sequencing as a novel tool for toxicogenomics

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Long read sequencing has recently been termed "method of the year 2022" by Nature. In particular, long-read transcriptome sequencing (LRTS) has a huge potential for toxicogenomics because of its ability to capture full-length isoform information. With LRTS drug-induced alternative isoforms and splicing events being causative or indicative of toxic processes can be detected and, thus, better biomarkers for risk assessment can be identified.

We have applied LRTS with the PacBio Sequel II Iso-Seq protocol on human hepatocytes treated with the HDAC inhibitor valproic acid (VPA). We showcase isoform identification and quantification as well as differential splicing analysis against untreated samples and compare the identified biomarkers to RNA-seq experiments on the same samples.

We exemplify the LRTS data analysis workflow with our novel computational tool IsoTools that, among others, provides statistical methods for differential splicing analysis. We highlight known VPA-induced splicing effects such as exon skipping in the FN1 gene and cryptic transcription start site formations as previously shown with other HDAC inhibitors. We demonstrate the power of LRTS by detection of VPA-induced complex splicing patterns such as mutual exon inclusions in the gene SLC39A14 gene and also by novel isoforms that have implications for liver diseases. Finally, we present *in vivo* relevance of the novel isoforms by validating their presence in human livers.

Our work shows that the complex transcriptome landscape can be resolved by LRTS in an unprecedented way and that LRTS presents a new method for refined biomarker identification in toxicogenomics.

Presentation: Oral

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Predictive toxicology using human iPS cells

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To improve the predictability in human, new approach methodologies (NAMs) has been expected to perform *in vitro* toxicology assessment in terms of animal alternatives testing. To date, various models using human iPSCs and mini organs that more closely mimic native tissue function have shown to be potential for practical applications. These *in vitro* methods can ensure our health and speed-up the review process. To accelerate the use of iPSC models, it is important that regulators, academia, and industry get together to discuss good cell culture practices using stem cells. In addition, sharing standardized protocols to assess the quality of differentiated models is an important process. To engage researchers in the exciting area, we have established a consortium of iPSCs to share datasets with positive and negative compounds and work together to develop new evaluation methods. As a result, we have demonstrated that iPSC-derived models have the ability to provide information on predictive toxicology. Recently, iPS cell-derived organoids and 3D culture systems have great attention as mini-organ models that recapitulate human tissue function. These 3D organoids need to be validated according to the context of use. By educational seminars and international meetings, the effective use of these advanced "fit-for-purpose" models requires continued education and practice. Here, I would like to present the current status of human iPSC models and discuss the future challenges for regulatory considerations.

Presentation: Oral

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Recent progress of alternatives to animal testing from regulatory science in Japan

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Regulatory science is an area where new approach methodologies (NAMs) are applied to the review process. In Japan, to extend healthy life expectancy, the Cabinet Office established a national top-down funding system AMED (Japan Agency for Medical Research and Development) from 2015 to promote consistent research and development from basic research to practical appli-

cation. In particular, regulatory science section in the government document clearly indicates that a new evaluation system using iPS cells is an important strategy for drug development.

Under this strategy, we have been working on the development and standardization of new *in vitro* pharmacological test methods and have successfully standardized cardiotoxicity evaluation methods in collaboration with international groups, such as FDA and HESI. More recently, the iPS cell project has expanded to include microphysiological systems, such as hepatotoxicity and the blood-brain barrier, and chemical safety issues. Developmental toxicity and *in silico* modeling are also important topics in animal alternatives.

Furthermore, to accelerate vaccine research and development, the SCARDA project was established within AMED at the end of March 2022 to provide safe and effective vaccines against emerging infectious diseases and to provide rapid and flexible funding in the event of a public health emergency. For example, *in vitro* infection assay systems has been developed for drug discovery and vaccine evaluation.

Thus, the promotion of alternative methods is an important issue in regulatory science, and it is expected that new test methods will be developed and standardized in the future.

Presentation: Oral

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Establishing scientific confidence in a cell-free method to predict decreased lung function

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Acute inhalation toxicity testing for regulatory purposes relies on the use of guideline studies in rodents. Apart from the ethical concerns of exposing animals to toxic chemicals, *in vivo* testing is expensive and time-consuming. Understanding the effect at the portal-of- entry, 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. In this presentation, 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 discussed. The method addresses the molecular initiating event of the adverse outcome pathway AOP 302; "inhibition of lung surfactant function" that can lead to the adverse outcome decreased lung function. The main function of lung surfactant is to regulate the surface tension at the respiratory air-liquid surface 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. On this background the current applicability domain of the method will be explored. Finally, a case study for the reproducibility within laboratory will be presented. 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.

Presentation: Oral

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Does preregistration of animal studies work? A plan to find out

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Building on lessons learned from clinical research, we recently established preregistration of animal studies as a pertinent intervention to improve transparency and study quality, yet no structured assessment has been performed to evaluate its efficacy.

Here we provide the first evaluation of the effect of preregistration on animal studies. Preregistered protocols and their corresponding publications were collected from the platforms Preclinicaltrials.eu and the Animal Study Registry, and compared with unregistered control publications based on journal-match (for similar article types) and author-match (to remove the team effect bias). Scoring of reporting quality using the ARRIVE guidelines essential 10 and internal validity using the SYRCLE's risk of bias tool will be performed blinded by two independent assessors to evalu-

ate the effect of preregistration. Each paper will receive an overall score between 0-1 corresponding to different quality categories: "Excellent" (0.8-1), "Good" (0.6-0.79), "Average" (0.4-0.59), "Mediocre" (0.2-0.39), and "Poor" (0-0.19), as inspired by validated methodology.

The project's pilot yielded promising results, showing that registered papers (n = 10) were better reported than the two comparator groups (n = 10 each), by scoring as "good", while controls scored as "average". From this pilot, we determined that only six papers per group were needed to reach sufficient power in the actual project.

The complete results of this project will be finalized in June 2023 and will provide the first evidence for the presence, or lack, of effects of preregistration, which should impact the perceived value of preregistration and its future implementation as a standard practice.

Presentation: Oral

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Fish and amphibian eleutheroembryo assays as alternatives to animal tests for regulatory assessment of endocrine activity of chemicals

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Regulatory assessment of endocrine-disrupting properties of chemicals relies upon extensive use of animals. For example, the recommended fish and amphibian tests require hundreds of animals per chemical to screen for endocrine activity, and two thousand to investigate adverse effects. OECD-validated eleutheroembryo assays have recently been introduced as New Approach Methodologies for the assessment of endocrine activity of chemicals in aquatic vertebrates. The Xenopus Eleutheroembryonic Thyroid Assay (XETA) allows for the detection of chemicals that are active on select pathways in the thyroid axis. Other eleutheroembryo assays in fish have also been validated by OECD for the estrogen and androgen axes (OECD TGs 250 and 251, respectively). The conditions for using these assays as alternatives to chronic fish *in vivo* tests still need to be defined. However, the use of the XETA as an alternative to the Amphibian Metamorphosis Assay in the context of (re)approval of pesticide active substances in Europe effectively resulted in ca. 50% reduction of animal use. Further animal (amphibian) reduction is expected from the validation of thyroid-specific endpoints in fish guideline studies. Though, the replacement of animal tests with eleutheroembryo assays ultimately relies on a thorough understanding of their applicability domains and acceptance by regulatory authorities, which varies amongst countries and jurisdictions. Discrepancies also exist amongst OECD eleutheroembryo TGs, which may complicate the practical implementation of these approaches. This and lessons learned from the use of eleutheroembryo assays over the last three years call for harmonization of the corresponding OECD TGs.

Presentation: Oral

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A "question-box" approach: Practical considerations for NAM-based agrochemical safety assessment

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This presentation will provide an overview of Industry perspectives on phasing in New Approach Methods for agrochemical products safety assessment. Current regulatory requirements consist of non-flexible data requirements, often consisting of vertebrate studies. Opportunities and challenges are faced when decision is to be taken related to integrated approaches for testing and assessment in the current paradigm. This entails aspects such as validation/qualification of methods for decision making, internal and external risk tolerance, challenges related to regulatory uptake and uncertainty assessment and perceived conflict of interest. A key aspect is how to move away from a "tick the box" list of studies to a "question box" approach. Case examples of human health safety assessment will be shown from acceptance of simple test guidelines and defined approaches developed for other classes of chemicals (skin sensitization), to more complex NAMs based read-across for existing classes of compounds (with particular example on reducing reliance on dog or rodent carcinogenicity bioassays), and complex high-tier risk assessment and hazard classification and labeling based on *in vitro* Points of Departure.

Presentation: Oral

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Next generation artificial intelligence-assisted tools for excelling regulatory acceptance, global harmonization and research evaluation in the life sciences

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Over the past decade, AI-assisted tools and workflows based on life science ontologies and databases have gained significant power, shaping strategic approaches to determine in scientific papers whether alternatives to animal testing such as *in vitro* assays, computer simulations, human-based studies and regulatory frameworks for human safety assessments have been considered.

In an international regulatory context, the development of Good *In vitro* Method Practices (GIVIMP) should be recognized as a top priority. Interoperability of artificial intelligence-assisted tools is essential for linking research data, best practices in evaluating chemical safety and experimental design, and establishing acceptance criteria and performance standards based on scientific evidence derived from *in vitro* datasets. The next generation of scientific research must adhere to clearly defined standard operating procedures to ensure rigorous and reproducible data, follow regulatory risk assessment processes, and promote education in animal-free science. In this regard, we analyzed artificial intelligence-assisted tools able to conduct a data extrapolation analysis, allow a faster interpretation of experimental entities, terminologies, and research applications, as well as streamline research evaluation processes based on their adherence to Good Method and Reporting Practices.

Case papers studies are presented to demonstrate the utility of the proposed framework while highlighting bias and uncertainty.

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Presentation: Oral

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Support of first-in-human clinical trials with human *in vitro* toxicity testing

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Standard preclinical models used for safety assessment of novel drugs sometimes present no or insufficient pharmacological activity, in particular with more recent therapeutic modalities (e.g., bispecific antibodies, gene and cell therapies). The EMA guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials encourages the use of *in vitro* studies with human-derived material, either as additional support or as an alternative to *in vivo* testing.

Under the umbrella of the safety reflection initiative of EFPIA's preclinical development working group (PDEG), a team of toxicologists from nine pharmaceutical companies started to compile current and future options for *in vitro* toxicity testing that will contribute to regulatory submissions relevant for first-in-human clinical trials. By defining different drug candidate categories with their specific safety assessment options, including alternative approaches to animal testing, the team is working towards provid-

ing guidance to increase the probability and address challenges for regulatory acceptance. In addition to challenges that will be highlighted, definitions of drug candidate categories will be presented together with respective examples of how *in vitro* risk assessment approaches were employed to complement or even replace standard animal models. These definitions and case studies should lead to a clearer path forward on how to meet regulatory expectations using alternative approaches to animal testing as appropriate safety assessment.

Presentation: Oral

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Leveraging *in vitro* transcriptomic points of departure in chemical safety evaluation

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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 target pathways and modes of action in a single screening assay. Researchers at EPA have applied this method to chemical screening studies of over 1,000 chemicals using multiple *in vitro* cell culture models to generate data that can be used for both hazard prediction and potency estimation, thereby informing risk assessments and prioritizing chemicals for further testing.

Assessing the reliability and reproducibility of *in vitro* HTTr screening methods is critical to their utility and adoption in regulatory applications. 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 more accurate potency estimates compared to concentration-response modeling of individual genes.

Advanced analysis methods are also needed to link gene expression changes to relevant chemical targets in order to predict organism-level hazards. We have developed novel methods to predict primary molecular targets based on HTTr data, which can then be linked to specific hazards and used to prioritize orthogonal follow-up testing with targeted assays. Overall, our work has shown that HTTr applied to *in vitro* cell lines provides a scalable and reliable toxicological screening method with potential utility to multiple use cases and regulatory contexts.

This abstract does not necessarily reflect US EPA policy.

Presentation: Oral

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Challenges and prospects for Colombia and Latin America in the evaluation of the safety of ingredients and cosmetic products

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In 2022, the National University of Colombia and UNIDO carried out the Safety Assessment of Cosmetic Products Training, to support this growing industry in Latin America. At the end, a panel was held with representatives of industry, organisms, academia, and the national regulatory body, who exposed their experience about safety of cosmetics (perceptions, concerns and actions to mitigate them and, challenges and strategies to assume them). Considerations on alternatives methods were stated. Since cosmetics have usually included well-characterized ingredients, they are safe under foreseeable use. However, it is challenging to evaluate safety of new biodiversity ingredients that are produced in the region. The ban on animal testing has reached Latin American countries such as Colombia without clearness on the alternative technics. Except for Brazil, there is not enough *In vitro* laboratories offer nor capacity to interpret the new approach methodologies. These tests are expensive and difficult to pay for small and medium industry, it is crucial make these affordable. Also, cosmetic users are unaware of replacement alternatives and when searching for "Cruelty Free" products they only want no animal testing and they ignore safety matter, this topic is obviated on cosmetics offered through social networks too; therefore, it is necessary to educate the consumer. Contrary to developed countries, new technologies for toxicity testing are not as reachable in Latin America, so

it is important to create and strengthen regional support networks that include cosmetic sector and experts in both security issues and alternatives to animal use.

Presentation: Oral

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Rat liver S9 incorporation into the reconstructed skin micronucleus assay to address scenarios of systemic metabolism

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The Reconstructed Skin Micronucleus (RSMN) assay has been recommended as an animal free follow-up test for skin relevant compounds found to be positive in standard *in vitro* clastogenicity assays. In the context of dermal exposure, human skin specific metabolism has shown to be accurately reflected in RS models, especially with extended exposures. However, scenarios may occur where substances penetrate the skin and undergo further metabolism in the liver. We therefore have evaluated the ability of rat liver S9 to complement the RSMN assay. Two approaches for S9 incorporation were examined: short 4 h exposure + recovery vs. continuous exposure. Excessive toxicity was observed using continuous exposure, therefore the 4 h S9 + recovery approach using 0.5% S9 was selected for further work. Cyclophosphamide (CP), a compound known to require metabolic activation to show a genotoxic effect, was tested at multiple concentrations and statistically significant and dose dependent increases in MN frequency were observed. In contrast, no MN increases were observed when comparable concentrations of CP were tested without S9. Work is ongoing to confirm and optimize the exposure scenario. In summary, these are promising results indicating that incorporation of 0.5% rat liver S9 can enhance the ability of the RSMN assay to detect compounds such as CP, which require liver-specific metabolism. This protocol is hoped to complement the current RSMN protocol in scenarios of expected systemic metabolism relevance.

Presentation: Oral

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Generating screening level developmental neurotoxicity (DNT) information of chemicals in a new approach methods (NAMs) battery

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Today 15-20% of children are diagnosed with a neurodevelopmental disorder. Evidence indicate that chemical exposure contributes to these disorders. However, majority of chemicals have not been tested for developmental neurotoxicity (DNT) as current test guidelines are based on traditional *in vivo* animal studies that are costly, time consuming and require large numbers of animals. Within the Division of Translational Toxicology, the DNT Health Effects Innovation (HEI) program was developed in 2019, to evaluate the risks of chemical exposure to the developing nervous system. One aim was to implement a DNT screening battery that covers key neurodevelopmental events to provide timely data for decision making and to prioritize compounds with potential for DNT. The battery includes 2D and 3D human and rodent *in vitro* assays that measure proliferation, cell migration, neurite growth, neural network formation and function, and a zebrafish embryo neurobehavior assay. Based on nominations from various stakeholders the DNT HEI program selected and distributed 115 chemicals for testing in the battery and additional 100+ chemicals currently undergoing testing. Moreover, the program developed a unified data analysis pipeline to combine data from the individual assays and DNT-DIVER, a web application tool. Combined with PBPK and IVIVE modeling, this approach was applied in the development of an IATA case study for the OECD DNT guidance document. It demonstrates applicability of the DNT battery for prioritization and how human exposure data can be used to interpret this data and support the contextualization of these studies in potential future risk assessment.

Presentation: Oral

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Progress towards reducing use of animals in chemical testing and adoption of new methods to evaluate the safety of chemicals and medical products in the United States

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In 2018, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) published the US Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States. ICCVAM is made up of representatives from US federal agencies that require or consider chemical safety testing data, and are interested in more rapid, human-relevant approaches to supplement or replace existing regulatory standard *in vivo* guideline tests. The National Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) provides scientific and operational support to ICCVAM through a variety of efforts including methods development and validation, construction of computational tools, communication and outreach, and stakeholder engagement, all driven by federal agency priorities and decision contexts. This talk will provide an overview of ICCVAM and NICEATM's progress in developing, evaluating, and implementing alternatives to animal testing in the five years since the publication of the roadmap. Key to the success of the roadmap has been implementation plans paired with specific toxicity endpoints, and communication with regulatory decision makers and end-users to understand stakeholder needs when designing and evaluating new methods. Efforts to replace the "six-pack" of acute toxicity tests and development of computational resources to support regulatory needs will be highlighted, as well as future challenges and opportunities in the areas of designing testing strategies for increasingly complex endpoints and flexible, fit-for-purposes approaches to establishing confidence in alternatives.

Presentation: Oral

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Human biological relevance: Topical toxicity case studies

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The concept of biological relevance is a key element in many modern scientific confidence frameworks designed to support flexible, fit-for-purpose approaches to validating and establishing scientific confidence in New Approach Methodologies (NAMs). The "gold standard" objective is to design NAMs that represent human biology in a way that exceeds the ability of reference *in vivo* guideline studies to predict specific toxicological effects, where possible. In the case of topical toxicity endpoints such as skin sensitization and skin/eye irritation, several decades of work have led to well-developed understanding of the mechanisms (e.g., via an Adverse Outcome Pathway (AOP)) and the relevant human anatomy and physiology. Test methods that map to key events or represent the critical biology with sufficient coverage can be assessed in terms of their human relevance and can even, in cases with robust human clinical data, be shown to predict the human outcome better than existing animal models when combined into defined approaches and integrated approached to testing and assessment. These approaches serve as benchmarks for development of future strategies to predict specific effects, particularly when tacking more complex endpoints whose mechanisms may need to be characterized via AOP networks or other analogous biological interaction maps.

Presentation: Oral

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Developing open-access datasets and predictive toxicology tools

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The successful adoption of predictive toxicology approaches requires high confidence curated reference data which are standardized to facilitate interoperability in drawing connections between toxicological endpoints and chemical properties through easily accessible tools. To effectively leverage predictive toxicology tools, it is imperative to identify and curate reference toxicological bioassay data as well as NAMs data within the appropriate context of use. Disparate data sources, however, can be difficult to aggregate and interpret. With a single open access point for interoperable data and predictive tools, computational toxicol-

ogy analyses can yield novel insights and pave the way to animal-free chemical assessments. Accessible tools with appropriately contextualized outputs can further facilitate interpretation and advance adoption of NAM-based evaluations. For example, approaches such as PBPK and IVIVE can help with dose-setting and target organ exposure predictions, while read-across and machine learning algorithms can provide insight into chemical property-based similarity, chemical use, and exposure predictions. By curating and harmonizing datasets, the Integrated Chemical Environment (ICE; https://ice.ntp.niehs.nih.gov/) provides users the opportunity to aggregate diverse data using a suite of tools to facilitate predictive analyses. As a centralized, well-documented, user-friendly resource for reference chemical lists, curated historical guideline toxicity test data, annotated NAM datasets, and powerful computational toxicology workflows and tools, ICE leverages NAMs and augmented intelligence approaches for next generation chemical assessments.

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AOP-based *in vitro* adversity to predict *in vivo* effects

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An adverse outcome pathway (AOP) is a framework for organizing information regarding the sequence of molecular and cellular events that can lead to a toxic effect when an organism is exposed to a substance, and, importantly, can provide a conceptual basis for non-animal testing strategies. Construction of an AOP can be used to demonstrate plausible links between biological interactions that describe how exposure to a substance might cause illness or injury and develop predictive models of cell- or biochemical-based tests that could be used to develop testing strategies for targeted toxicity. An AOP that represents the underlying signaling pathways leading to an adverse *in vivo* effect, e.g., skin sensitization, can be used as a foundation upon which *in vitro* test methods are mapped and combined, and thus, *in vitro* responses may serve as a measure of that adverse effect. Though non-animal testing strategies do not always require an AOP, the use of these frameworks have contributed to regulatory acceptance of *in vitro* adversity measures used for hazard identification and characterization (including classification and labeling) without having to demonstrate the effect *in vivo*. While this approach is not feasible or practical for all toxicity endpoints, there are a number of examples where detailed AOP frameworks may support an *in vitro* determination of adversity.

Presentation: Oral

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AI on the CompTox continuum: Applications in environmental chemical assessment

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The field of computational toxicology has enjoyed rapid growth over the last decade, with the maturation of cognitive algorithmic tools and software to mine, process, and model data to facilitate robust and reliable predictions of chemical property, activity, and toxicity endpoints. Success lies in iterative and mutually informative approaches along a continuum of FAIR (findable, accessible, interoperable, and reusable) data resources, predictive analyses, experimentation, and mechanistic models, with the goal of generating insights into human disease processes and their susceptibility to environmental perturbations. Underpinning this "CompTox continuum" is augmented intelligence, a field that leverages big data and computational tools (e.g. ICE: https://ice.ntp.niehs.nih.gov/) to join techniques of machine learning, artificial intelligence, natural language processing, mathematical modeling, and data analytics to enhance and support human intellect. Applications of augmented intelligence in environmental chemical assessments that will be discussed range from models for specific toxicity endpoints (e.g. endocrine disruption, cardiovascular toxicity), to automation of published literature curation and annotation, to systems modeling approaches and computational workflows enabling hypothesis generation and testing.

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A SafetAI Initiative: AI based prediction initiative to assist reviewers with predicting toxicity endpoint

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Drug safety is of great concern to public health. In addition, during the Investigational New Drug (IND) application submission process, the FDA specifically reviews the safety of the submitted drug candidate before the sponsor can initiate any clinical trials. SafetAI is a collaborative initiative led by CDER (e.g., funded by a FY2022 CDER Safety Research Interest Group grant), where

NCTR is developing a suite of deep learning-based QSAR models for various safety endpoints critical to regulatory science and the IND review. Currently, the initiative has focused on five key safety endpoints: hepatotoxicity, carcinogenicity and mutagenicity. We are developing a novel deep learning-based precision system for toxicity (DeepPST) which is designed to optimize toxicity prediction for individual compounds based on their chemical characteristics. In a pilot study, DeepPST was compared to several conventional machine learning and state-of-the-art deep learning methods for predicting drug-induced liver injury (DILI), carcinogenicity, and Ames mutagenicity. The preliminary results from DeepPST yielded significant improvement in these toxicity endpoints in comparison to other deep learning and QSAR methods. SafetAI facilitates drug-safety research with the novel DeepPST architecture that improves the "precision" in toxicity assessment by tailoring prediction to chemical characteristics. It could play a role in providing critical safety information during the IND review process.

Presentation: Oral

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An animal component-free human neuroprogenitor cell culture model for high-throughput chemical hazard screening

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The US EPA developmental neurotoxicity *in vitro* testing battery (DNT-IVB) includes proliferation and apoptosis assays using human hNP1 neuroprogenitor cells. In addition, US EPA has proposed a tiered hazard evaluation strategy that uses high-throughput transcriptomics (HTTr) and high-throughput phenotypic profiling (HTPP) to characterize the biological activity of chemicals in human-derived cells, including hNP1. Currently, for both Tier 1 and DNT-IVB screening, hNP1 cells are cultured on mouse-derived laminin (MDL) that is isolated from mouse Engelbreth-Holm-Swarm sarcoma tumors. While effective in supporting the growth of hNP1 cells, MDL can vary by lot. The goal of this work was to identify a human-derived recombinant laminin (HDL) for hNP1 cells that can replace MDL in the Tier 1 and DNT-IVB assays. Three concentrations (1, 5, 10 µg/mL) of four types of HDL (BioLamina LN111, LN211, LN511, LN521) were evaluated as growth substrates at 48 hours in 384-well format. Transcriptomic gene set variation analysis of hNP1 cells on each HDL type indicated that cells cultured on LN111 had the greatest enrichment for embryonic neural stem cell markers. Principal component analysis indicated that HTPP profiles for all HDL conditions were distinct from MDL with LN111 being the most similar based on centroid distance analysis. LN111 was selected for HTPP chemical screening studies to facilitate comparison to previously generated screening data from hNP1 cells using the MDL substrate. This work demonstrates that LN111 may be a suitable replacement for MDL in the Tier 1 and DNT-IVB assays. This abstract does not reflect US EPA policy.

Presentation: Oral

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ICCVAM activities 5 years into the Strategic Roadmap: Successes and opportunities

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The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is made up of representatives from US federal agencies that require or consider chemical safety testing data, and are interested in more rapid, human-relevant approaches to supplement or replace existing regulatory standard *in vivo* guideline tests. The National Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) provides scientific and operational support to ICCVAM through a variety of efforts including methods development and validation, construction of computational tools, communication and outreach, and stakeholder engagement, all driven by federal agency priorities and decision contexts. This talk will provide an overview of ICCVAM and NICEATM's progress in developing, evaluating, and implementing alternatives to animal testing in the five years since the publication of the US Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States. Successes, such as efforts to replace the "six-pack" of acute toxicity tests and development of computational resources to support regulatory needs, will be highlighted, as well as future challenges and opportunities in the areas of designing testing strategies for increasingly complex endpoints and flexible, fit-for-purposes approaches to establishing confidence in alternatives.

Presentation: Oral

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Advancing science and ethics through legislation: The HEARTS Act

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The field of alternatives research has accelerated in the past 30 years, largely because of legislative pressures on specific sectors to end animal testing and/or use non-animal methods. However, Office of Inspector General reports have repeatedly note repeated failures to search for alternatives to painful procedures and to document the availability of alternatives in research proposals. In 2019, the United States Government Accountability Office (GAO) recommended that federal agencies better monitor and report on their efforts to develop and promote replacement alternatives and decrease animal use.

The US National Institutes of Health (NIH) is experiencing a growing demand from Congress and the public to replace the use of animals in publicly funded research. Our presentation will guide you through the Humane and Existing Alternatives in Research and Testing Sciences (HEARTS) Act which is set to advance science and ethics by prioritizing the use of non-animal methods in NIH funded research. This will be achieved in several ways including directing the NIH to incentivize the use of non-animal methods, establishing guidelines for alternatives searches and requiring that proposals are reviewed by at least one person with expertise in non-animal research methods. In addition, the Act establishes a "National Center for Alternatives to Animals in Research and Testing" dedicated to the funding and development of alternatives. The new Center would also track the number of animals used in federally funded research and be tasked with developing a plan for reducing those numbers.

Presentation: Oral

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Critical dialogue for evolving NAM support and policy in South Korea

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Political and public campaigns, along with collaborative activities with regulatory agencies are paving the way for regulatory uptake of non-animal methods (NAMs) in Korea. Following enactment of the European cosmetics ban, animal protection (AP) groups began a campaign to apply societal and political pressure for the Korean government to follow suit, resulting in the Cosmetics Act amendment prohibiting animal testing in 2015. This stimulated research interest in NAMs focused on cosmetics. Pressure by international companies and AP groups continued, resulting in Korean chemical law amendments passed 2018 and 2020 that support the use of NAMs. After further campaigns to convince industry and regulatory agencies to invest in NAMs, the Ministry of Environment (MoE) introduced the 2030 vision for chemical safety and animal welfare that promises to vastly increase the use of NAMs by 2030. Our discussions with MoE resulted in creation of a task force dedicated to a roadmap for NAM development and implementation in which we participated. Despite increasing interest in research and development of NAMs, the lack of connection between research funding authorities and regulatory agencies remains a barrier to the uptake NAMs at the regulatory level. To support on-going regional efforts and compel international, multilateral collaboration, discussions between KoCVAM, politicians, NGOs, research and industry experts resulted in two bills being introduced. These bills will provide structural and financial support for NAMs at multiple levels. The evolution of multi-stakeholder dialogue in Korea has resulted in a rapidly evolving international voice to move towards non-animal advanced testing and approaches.

Presentation: Oral

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Young TPI: Empowering young people to go animal testing-free

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The next generation of young professionals and students is essential to accelerate the transition towards animal-free science. The initiative Transitie Proefdiervrije Innovatie (TPI), therefore, created Young TPI to involve the youth and make them aware of the transition and what is possible in the field of animal-free innovations. TPI is comprised by the Dutch government, companies and academia, and shifted focus in 2018 from reducing animal studies to increasing animal-free innovations.

Young TPI is a network aiming to empower the new scientific generation to use their thinking and acting power in relevant new

animal-testing-free initiatives and existing forms of consultation. The network is active in the breadth of research, from fundamental, translational, and regulatory research to education, to find out how to advance the acceleration towards animal-free methods. It also focuses on understanding the factors behind the acceptance and implementation of animal-free methods. It accomplishes this through three pillars: stimulate the transition (e.g., working with collaborators, ambassadors, and creating incentives for a change), raise awareness & show the possibilities (with lectures and courses) and create a network & share experience.

Starting in February 2022, the network attracted 150 young professionals and students. The network currently focuses on the Netherlands, and organised activities such as physical lectures about transitions, a webinar about systematic reviews, a think tank for the ZonMw Knowledge Agenda, the Transition Challenge and network events. Young TPI intends to expand internationally in the upcoming years to empower all young scientists to go animal-testing-free.

Presentation: Oral

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Using zebra fish for chemical mixtures risk assessment

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For decades toxicologists relied solely on rodent-based assays to assess the toxicity/safety of chemicals, but the cost of these assays continues to limit thorough safety assessments, particularly for chemical mixtures. Although individual models are insufficient to meet these challenges, the complexity of mixtures must be met with the sensitivity to quantify the toxicity of chemicals with similar or different modes of action. We have demonstrated that the intrinsic advantages of zebrafish make this model ideal for filling important knowledge gaps. Multi-dimensional zebrafish assays provide rapid ways to discover and compare the bioactivity of chemicals and mixtures for decision making and as a path to define mechanisms of action. This presentation will provide examples where high throughput screening and systems approaches can quickly define the toxicity of individual chemicals, component mixtures, and complex whole mixtures. Zebrafish are typically exposed test chemicals or mixtures across concentrations spanning orders of magnitude, with deep biological replication allowing for rigorous statistical analysis. Chemical effects on survival and development are evaluated at 24 and 120 hpf, and motor behavior changes are measured using two high throughput photomotor response assays. Chemical exposures produce distinct biological activity patterns, and we routinely collect unbiased whole-genome transcriptomic responses to discover the expression changes that are causally linked to phenotypic responses produced by the exposures which should improve data translation. The generation of phenotypic and gene expression response data from structurally diverse chemicals and mixtures a scale necessary to begin to predict the structural attributes that result in biocompatibility versus toxicity.

Presentation: Oral

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From academia to the shopping cart: How to use new methods in real life chemical risk assessment

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The number of available new methods based on cell systems and *in silico* methods is increasing fast. The potential and need to invest in such approaches are well represented by the financial efforts dedicated to the development of Novel Approach Methodologies (NAM), i.e. with the H2020 funded EU-ToxRisk project (ended in 2021) and the ASPIS cluster. Nevertheless, NAM application in real-life chemical assessment is still limited. One of the reasons is the industry's difficulties to move from the standard animal-based testing when dealing with regulatory requests or other toxicological questions. While Contract Research Organisations (CROs) have long experience with animal tests, the application of non-animal approaches is limited. To solve this problem, a team from the EU-ToxRisk project initiated the SaferWorldbyDesign platform (https://saferworldbydesign.com/) now continuing within the RISK-HUNT3R project. The platform's mission is to have a role in the transition toward a safer world by translating research methods developed into practical applications through a one-stop-shop. The service includes the identification of the best solution for the specific problem, whether it is the design of safer products or the compliance with regulatory requirements, the management of the testing experiments in the most suitable laboratories until the preparation of the final report that contains practical indications on how to present the results. Thanks to its involvement within the ASPIS and the cluster's wide stakeholder network, the platform will increase its impact with the goal to ensure optimal conditions, in terms of regulatory and commercial objectives.

Presentation: Oral

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Partnering with policymakers to advance NAMs: Strategies and best practices that achieve results

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Advancing new approaches for use in research and testing requires a comprehensive strategy that includes scientific support, education and outreach, and policy change. Policymakers play a crucial role in supporting innovative science that offers the potential to improve research and testing while reducing and replacing animal use. In recent years, scientists and lawyers have partnered with policymakers on a multitude of initiatives that are propelling science forward by supporting the use of new approaches. Many policymakers are now eager to work on initiatives that improve science and protect animals. Advances include legislation requiring agency and industry use of nonanimal approaches, funding allocated to support reduction of animal testing, and requests to update regulatory requirements for animal use. But, how did we get here? Relationship building, and the ability to effectively communicate the importance of ethics and scientific advancement, lay the foundation. Successful endeavors also require persistence, expertise, and creative solutions to the barriers that scientists face in developing and using new approaches.

Presentation: Oral

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Aggression in group-housed male mice: A dual approach to increased understanding

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Aggression among group-housed male mice is a major animal welfare concern often observed at animal facilities. Pain, stress, and social isolation, as a consequence of aggression, can alter physiological parameters creating variability and jeopardizing scientific validity.

The Swedish National Committee for the Protection of Animals Used for Scientific Purposes and the Swedish 3Rs Center collaborated with experts to map the problem of aggression in group housed male mice at university research facilities and private companies in Sweden. Veterinarians, ethologists, animal technicians and researchers participated in eleven workshops and one survey. The results have been communicated at different meetings and compiled in national guidelines. Furthermore, we performed a systematic literature review to map how the literature support, or do not support, published recommendations within the field.

The compiled results suggest keeping stable groups, using less aggressive strains and enrichment as important factors to prevent aggression. Hiding devices and nesting material seem to prevent aggression better than locomotor devices that could be monopolized. However, there is no one-solution-fits-all and different interventions can work at different facilities. The results from the workshops and survey also highlight the importance of establishing and evaluating routines at the facilities, and to promote collaboration and communication between researchers, veterinarians, animal technicians and the management.

Both approaches – a literature review and compiled experiences from research facilities, proved to be important and to complement each other in developing guidelines on how to prevent aggression in group housed male mice.

Presentation: Oral

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Every day with every interaction: Developing a common culture across disciplines

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Merriam-Webster defines culture in part as "the set of shared attitudes, values, goals, and practices that characterizes an institution or organization." Changing animal handling practices toward more cooperative techniques requires change at a cultural level in research settings. At the Washington National Primate Center (WaNPRC), our animal training program has established a core set of values based in positive reinforcement. We have taught these values as well as a common language for interacting with monkeys to everyone, across all disciplines, who interact with our animals. A substantial increase in the number of requests to train cooperative behaviors over the past 5 years is evidence that the culture change is taking effect. Currently we train fifteen distinct behaviors across

disciplines. Success is also reflected in the number of people with the skills to effectively elicit these behaviors, with 71 people who can train at least one of those 15 behaviors. Whether Neuroscience, Infectious Disease, or Bioengineering, every scientific core at the WaNPRC requires a certain level of cooperation from our non-human primate subjects. The tasks required from the animals for each of these disciplines is different, however the basic tenets of behavior shaping and the use of positive reinforcement to elicit cooperative behaviors is the same. By giving everyone who interacts with our animals a common knowledge base and language for communicating with the primates and each other, we have shifted the culture towards the use of positive reinforcement and cooperative handling.

Presentation: Oral

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Regulatory acceptance and implementation of new approach methodologies in Taiwan

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Being Aware of the global developments on 3Rs and new approach methodologies (NAMs), National Health Research Institutes (NHRI) in Taiwan has involved in promotion and implementation of internationally validated NAMs and strategies since 2017. In 2020, NHRI represented Taiwan to join the International Cooperation on Alternative Test Methods as a partner. Meanwhile, Taiwan governmental agencies have made some progresses on regulatory acceptance of NAMs. Taiwan Food and Drug Administration bans animal testing for both cosmetic products and ingredients since 2019. In 2020, Taiwan Environment Protection Administration begins to review and accept the submitted data generated with ISO or OECD announced NAMs or *in silico* methods, for toxic endpoints including skin and eyes adverse effects as well as aquatic toxicity. In 2022, Taiwan Council of Agriculture adopts non-animal methods for production and quality control of classical swine fever virus vaccine. More efforts are in progress for evaluating NAMs in registration of new chemicals and pesticides. The Taiwan 3R Office was officially set up in 2022. An interagency platform is launched to develop more NAMs for biomedical research.

Presentation: Oral

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Overcoming challenges in the development of quantitative AOPs

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The adverse outcome pathway (AOP) is a framework to organise key events (KE) and KE relationships (KERs) across multiple levels of biological organisation, from the molecular initiating event to a toxicologically relevant adverse outcome. By necessity KERs include an assessment of dose-concordance between KEs. However, most AOPs are qualitative and therefore uninformative for the derivation of a point of departure to use in risk assessments. Quantitative AOPs (qAOPs) are a tool for a biologically based prediction of the quantitative exposure of a chemical needed to produce an adverse outcome. A range of mathematical modelling approaches are available to produce qAOPs. There is a need for guidance on the building, practical application, and validation of

qAOPs. Therefore, the European Centre for Ecotoxicology and Toxicology of Chemicals invited experts from different sectors to make recommendations on the design, interpretation, and application of quantitative KERs, based on existing qualitative AOPs that are provide confidence for in use in regulatory decision making. They considered (1) What level of biological detail is necessary to include in a qAOP, (2) what is the most appropriate modelling approach, and (3) how do we ensure the quality and accessibility of qAOP models and their prediction? Briefly, the mathematical model needs to be "fit-for-purpose" and that specific purpose must be defined at the start. All modelling methodologies require the identification, extraction and use of reliable data and information to inform this process. Recommendation ways to achieve these goals will be shared.

Presentation: Oral

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Reducing the number of controls in fish early-life stage toxicity tests when solvents are required

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This project investigates the feasibility of using only the solvent control and omitting the water control when a solvent is used in the fish early-life stage toxicity (FELS) test. The use of only one control would substantially reduce the number of fish and resources used in tests for which solvents are required.

A FELS database including solvent and water controls and concentration-response data was developed to determine whether there are systematic differences between the water and solvent controls. For each response, the distributions of control data (means, between- and within-replicate variances) for water, solvent, and pooled controls were investigated. The FELS concentration-response data were used to investigate how the NOEC and EC10 depend on the choice of control. The power or predictive value of the analyses comparing the choice of control was investigated using statistical simulations.

Statistical simulations indicate that for the solvent dimethylformamide, fathead minnow EC10 estimates for length and weight based on the solvent control are more accurate than those based on either the water or pooled control only. There was a greater ability to fit models for ECx estimation with the solvent control versus the water control. Use of the solvent control maintains 80% power to detect 10% effects with lower false positive rates than other control choices.

Therefore, there is preliminary statistical evidence to support omitting the water control in FELS studies using a solvent.

This presentation reflects the views of the authors and should not be construed to represent US FDA's views or policies.

Presentation: Oral

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Target images and their role in facilitating the transition towards non-animal science

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The Netherlands National Committee for the protection of animals used for scientific purposes (NCad) published its policy advice "Transition to non-animal research – On opportunities for the phasing out of animal procedures and the stimulation of innovation without laboratory animals" in 2017. It contains recommendations for accelerating the transition to animal-free research and education. One of the recommendations was to create target images for basic/fundamental research. Target images describe clear transition objectives for a specific research domain aimed at reducing the use of laboratory animals with equal or better research quality and define the prerequisites that must be met to achieve the targets. The typical horizon for a target image is 5 to 10 years. Currently, four target images are in different stages of realization. The target image for the Neurosciences was published in 2021, and the one for Education and Training at the end of 2022. The target images for cardiovascular research and immunology are expected to be published in the second half of 2023. See also abstract NAMs: target image immunology.

Target images are drafted and published by the research domains themselves. By setting ambitious and realistic targets and detailing prerequisites, a research domain takes its responsibility to accelerate the transition to animal-free research. The concept of target images with examples from the published target images will be presented.

Presentation: Oral

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Bringing reproducible research and programming skills to large research consortia; think big, act small

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Volume, velocity, variety and veracity of data in Life Sciences research is increasing. This leads to an increase in the development and implementation of computational approaches in research projects. Many of these research projects are organized as consortia with several partners, which chip in to execute the work. These consortia typically have a range of expertise covering life sciences-related fields. The need for delivering reproducible research and well documented, tested software applications in these projects is high, but expertise to achieve this is often lacking. Reproducible research covers the steps from obtaining data to the final product. Although the skills required for conducting research in a reproducible manner are well-documented, they are often not effectively implemented. They include programming for data science, data management (FAIR data), adopting workflows and software development. In this work, we present our educational program for bringing reproducible research to two projects: 1) The EU Horizon 2020 project ONTOX and 2) The Dutch VHP4Safety project. The program aims to increase expertise for doing research in a reproducible manner to both early career and more advanced researchers. We have created an open collection of learning materials, available to all participants of the courses and beyond. Here we present the layout and content of the individual courses, their integrated aim, and the underlying didactics. We share our lessons learned and prevent common pitfalls when providing these kinds of trainings. The program is evolving constantly due to the community-driven approach and will serve as a legacy of both projects.

Presentation: Oral

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Decisions, decisions

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This talk plus discussion will focus on how to educate new researchers about both getting the experimental unit for a particular experiment right and deciding on a suitable blocking arrangement to give an efficient and reproducible experiment. Deciding on the experimental unit is not a simple matter as Lasic et al., 2018 have cogently argued in "What exactly is 'N' in cell culture and animal experiments?" [1]. It determines the number to put into a statistical test and overestimating the true N can greatly overestimate the power of an experiment. Deciding on a good blocking design is also a skill new researchers need to develop to make good use of resources and enhance reproducibility. For both animal and *in vitro* studies correct design of an experiment and analysis of the results is important for confidence in its validity and public acceptance of scientific and toxicological findings. So how to identify the experimental unit correctly and how to set up a good, randomised block design are key topics for experimental design teaching. Members of the FELASA Experimental Design Working Group have developed teaching material which has proved effective in helping understanding of the issues. These issues will be introduced in a short talk and then participants given the teaching material to discuss and comment. The presentation is particularly for those educating new researchers or who are coming into teaching or judging experimental design for biomedical studies, both *in vivo* and *in vitro*.

Reference

[1] *PLoS Biol 16(4)*, e2005282.

Presentation: Oral

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Critical needs for non-animal regulatory hazard assessment: A REACH perspective

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The needs for NAMs are different, depending on the type of chemical regulation and geographical region. NAMs to support the screening, prioritisation and read-across are available, continue to be developed and are used to some extent by regulators around the

globe, in line with the legal frameworks and regulatory requirements. However, there are currently only a few endpoints where full replacement of *in vivo* studies with non-animal methods has been accepted in a way that is suitable for classification and labelling or to conclude on (no)hazard. This is the case for the endpoints where a particular adverse effect can be addressed and the mechanism(s) leading to it are in general well understood (e.g., skin sensitisation). For the other, more complex toxicological endpoints (e.g., reproductive toxicity) the animal testing is still needed to characterise the hazard in line with the requirements of the GHS and CLP.

This presentation will identify, from a conceptual and scientific perspective, the critical needs for moving towards an animal free system for hazard assessment, in line with the regulatory requirements in Europe, which rely on REACH for data generation. It will build on ECHA's own experience, and the feedback expected from the stakeholders at the NAM workshop to be organised in Helsinki in June 2023. Case studies to benchmark NAMs developments are essential and will be discussed together with other critical needs such as better focus of research relevant for regulatory purposes, standardisation and validation needs.

Presentation: Oral

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Non-animal methods in neuroscience – Focus on Alzheimer's and Parkinson's disease

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One of the major challenges facing Europe is its ageing population and the associated increase in cases of neurodegenerative diseases such as Alzheimer's disease (AD, the most common type of dementia) and Parkinson's disease (PD). AD affects over 8 million people in Europe, and this figure is expected to double in the next 30 years. Population prevalence of PD, the second most widespread neurodegenerative disease, increases from about 1% at age 60 to 4% by age 80.

Existing therapies for these diseases are limited, and typically only treat the symptoms rather than providing a cure. Although there has been considerable investment in research, based predominantly on animal models, progress in discovering and approving effective treatments has been low. Indeed, the overall failure rate of drug development for these two diseases is particularly high (99% for AD), with one explanation being the poor translation of research in animals to humans.

For this reason, EURL-ECVAM launched a study to collect current and emerging non-animal models and approaches in use for basic and applied research in the field of AD and PD, with the aim to provide an inventory and scientific evaluation of innovative (human-based) models.

We reviewed more than 13,000 manuscripts and identified 568 advanced non-animal approaches mainly based on simple cell cultures, 3D cultures/organoids and brain-on-chip technologies. These can be exploited to provide strong mechanistic rationale for diagnostic, preventative and therapeutic interventions, for further reducing the number of animals employed in neuroscience, and ultimately increase translatability for the benefit of patients.

Presentation: Oral

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Helping animal-free innovation cross the Valley of Death: Empowering young researcher to take the leap from lab to business

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Background: Creating strong ventures based on animal free innovation helps it reach end users which can increase the use of non-animal methods. Early career researchers often wonder how to take their research to the next level. Enter the Proefdiervrij Venture Challenge, a training program that helps young (and old) researchers take their research towards broader implementation.

Method: The Proefdiervrij Venture Challenge is a 3-month program containing multiple workshops, 1 on 1 training sessions and feedback from experienced entrepreneurs. The end product is a business plan that is presented in front of a jury and a panel of investors. The jury chooses a winner, who wins 25.000 euros to be used for further research and development of the model or product. While the investors might decide to invest in one of the teams based on their pitch, so everyone could be a winner.

Results: Three editions were held since 2020, with 12 teams participating. Although data on success rates is still being gathered, based on feedback from interviews and questionnaires, we can already say that the Proefdiervrij Venture Challenge presents a valuable learning opportunity that provides new skills which can be directly implemented in daily practice ("Participation in the Proefdiervrij venture challenge equipped us with insights we wouldn't have gained otherwise"). Especially for young researchers, it can serve as a guide for the next phase in their career ("Each

exercise was designed to take us a step further towards translating strong science into an even stronger venture").

Presentation: Oral

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Robotic device for fully automated high-content screening on C. *elegans* as a novel NAMs platform for chemical toxicity assessment

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Nematode Caenorhabditis elegans constitutes a valuable New Approach Method (NAM) for multiple applications, including predictive toxicology. This microscopic worm gained popularity for its ideal short size and life cycle, ease of cultivation and propagation, and powerful genetic toolkit. While *C. elegans* has the potential to complement *in vitro* models to better predict toxic outcomes in mammals, the current experimentation methods lack automation and standardization, limiting their wider use in screenings.

In response, we developed a microfluidic-based robotic platform that automates the entire process of *C. elegans* culture, treatment, high-content imaging, and phenotypic analysis. The platform is able to execute multiple toxicity assays, including the possibility of using the existing sample collection of reporter strains thanks to the fluorescent imaging capability.

As an illustration, we evaluated the reproductive and developmental effects of twenty benchmark chemicals on *C. elegans* using the proposed platform. Synchronized populations of worms were chronically exposed to five doses of test compounds starting from the last larval stage (L4). Time-resolved phenotypic readouts were automatically extracted from the hourly-collected images of the worms, including growth dynamics, sexual maturity, fertility, embryonic viability, progeny accumulation and survival rate. Out of the tested compounds, methotrexate showed the most pronounced embryonic viability adverse effects, while bisphenol A strongly impacted the mothers' development.

Overall, we propose an innovative solution for rapid identification of toxic compounds and their mechanism of toxicity, bridging the gap between *in vitro* and *in vivo* assays. Our technology allows not only endpoint measurements' collection, but also the monitoring of biological responses' dynamics.

Presentation: Oral

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Evolving regulatory frameworks require methods that are both innovative and scientifically valid

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The EU has recently put forward a large-scale initiative to address climate change and environmental degradation called the European Green Deal. The aim of the Green Deal is for Europe to become a sustainable climate neutral and circular economy by 2050. One of its goals is to better protect human health and the environment by addressing pollution from all sources and move towards an environment that is essentially free from toxicants. A key element under this goal is the European Commission's chemicals strategy for sustainability. This strategy sets out more than 80 actions including the promotion of the development of international standards and innovative risk assessment tools, notably with the OECD, and promotion of their use under international frameworks to shift further away from animal testing.

If done right, validation of new scientific tools and harmonization of testing practices can accelerate the pace of chemical safety testing and assessment, reduce the use of laboratory animals and better protect human health and the environment. This presentation will explain, through examples, how validation practice is evolving to respond to regulatory and policy needs.

Presentation: Oral

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A worldwide survey on the use of animal-derived materials and reagents in scientific experimentation

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The use of cell and tissue-based methods has been increasing exponentially while still routinely containing animal-derived components, including serum, coating materials, growth factors and an-

tibodies. In addition to ethical concerns, the use of animal-derived materials and reagents may compromise reproducibility, generally associated with presence of undefined components and batch-to-batch variability [1,2]. However, non-animal materials, such as chemically-defined media or animal-free recombinant antibodies, are becoming increasingly available. Their use is encouraged by EU's Directive 2010/63 and OECD's Good In vitro Method Practices (GIVIMP) [3].

To map the current state of use of animal-derived reagents across different sectors and to identify obstacles possibly hampering the implementation of non-animal-derived alternatives, a global online survey was conducted. It was addressed to scientists working on *in vivo*, *in vitro*, *in silico* methods, in academia and industry, to understand: 1) the most commonly used animal-derived materials, 2) the main issues associated with production and use of animal-derived materials, 3) the current level of knowledge on available non-animal-derived materials, and 4) what educational and information sources could be most useful or impactful to disseminate knowledge on non-animal-derived replacements.

Here we provide an overview of the survey replies [4] and discuss possible proposals to increase awareness, acceptance and use of non-animal-derived ingredients.

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Presentation: Oral

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Using OECD QSAR toolbox to support regulatory safety assessments

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The OECD QSAR Toolbox can be used to support hazard assessment and minimize animal testing in chemical safety assessments for regulatory application. After an overview of the program, we describe several examples of how to use this tool in a variety of real-life regulatory applications.

The QSAR toolbox is a freely available computer program developed by the European Chemicals Agency (ECHA) and the Organisation for Economic Co-operation and Development (OECD). The software supports the hazard assessment of chemicals in a reproducible and transparent way; promotes the use of new approach methodologies (NAMs) to minimize unnecessary animal testing without reducing the safety of human health and environment; and provides mechanistic, physicochemical and other useful information about chemical substances. Several examples of how to use this tool in a variety of real-life regulatory applications, e.g., using the automated workflow for Defined Approaches for Skin Sensitization in place of the *in vivo* murine local lymph node assay, identifying analogues and filling data gaps by read-across for simple and complex endpoints like *in vitro* mutagenicity and developmental toxicity and how to categorize large datasets according to a common mechanism of action.

This talk will demonstrate the utility of the OECD QSAR Toolbox in fulfilling protective regulatory safety assessments whilst reducing animal testing.

Presentation: Oral

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Development of a non-animal integrated approach to testing and assessment for acute aquatic toxicity for classification and labelling

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Information on acute toxicity of several species is generally required as part of an ecotoxicological assessment. Within regulatory toxicology, three trophic levels are typically considered as a proxy of the ecosystem: fish, daphnia and algae. Acute effects are typically studied using one or more OECD guideline standardised tests including the Fish Acute Toxicity Test (OECD 203); the Fish Embryo Acute Toxicity Test (OECD 236) and the Fish Cell Line Acute Toxicity – The RTgill-W1 cell line assay (OECD 249). For animal welfare reasons as well as the quest for increased relevance, biological coverage and throughput, there have been significant efforts to reduce or eliminate the number of vertebrates, namely fish, used in ecotoxicological regulatory assessment by applying the 3Rs principle, e.g., replacing *in vivo* tests with *in vitro* assays, or by developing Integrated Approaches to Testing and Assessment (IATA). To build upon this body of work, a modular non-animal IATA has been created, using several components including *in silico* predictions, physicochemical parameters and mode of action data.

A high-quality dataset of existing *in vivo* and *in vitro* data was compiled, and the *in vivo* data used to develop a multi-class quantitative structure activity relationship (QSAR) model. The IATA is designed to provide a categorical output suitable for use within the EU Classification, Labelling and Packaging (CLP) and United Nations Globally Harmonized System of Classification and Labelling (UN GHS) frameworks. Case studies and considerations with respect to species relevance and applicability domain will be discussed within.

Presentation: Oral

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EPAA project: Use of new approach methodologies (NAMs) in regulatory decisions for chemical safety

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The European Partnership on Alternative Approaches to Animal Testing (EPAA) includes 5 Directorates-General of the European Commission, 3 EU Regulatory Agencies, 37 Companies, and 8 European industry federations. In Nov 2021, EPAA held a 2-day workshop to exchange information between EPAA partners on how New Approach Methodologies (NAMs) are being used/considered for regulatory use in safety assessment and registration. The discussion involved NAM users from across EPAA's Industry and European Commission partners talking about their experiences to inform future EPAA activities on the use of NAMs for decisions on chemical safety.

a) Are there circumstances where NAMs could be used for chemical safety assessments or to provide information for the classification and labelling of ingredients in the EU – regardless of tonnage?
b) How could NAMs be used to provide alternative Derived No-Effect Levels (DNELs) for decision-making?
c) Could a and b contribute significantly to the development of the EU Chemicals Strategy for Sustainability?

The output from the workshop was published in 2022 [1]. The conclusions of the workshop and follow-up activities by the EPAA to establish a "NAMs User Forum" and work to explore the use of NAMs for systemic toxicity classification will be discussed.

Reference

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Presentation: Oral

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Key elements for achieving a transition to non-animal science

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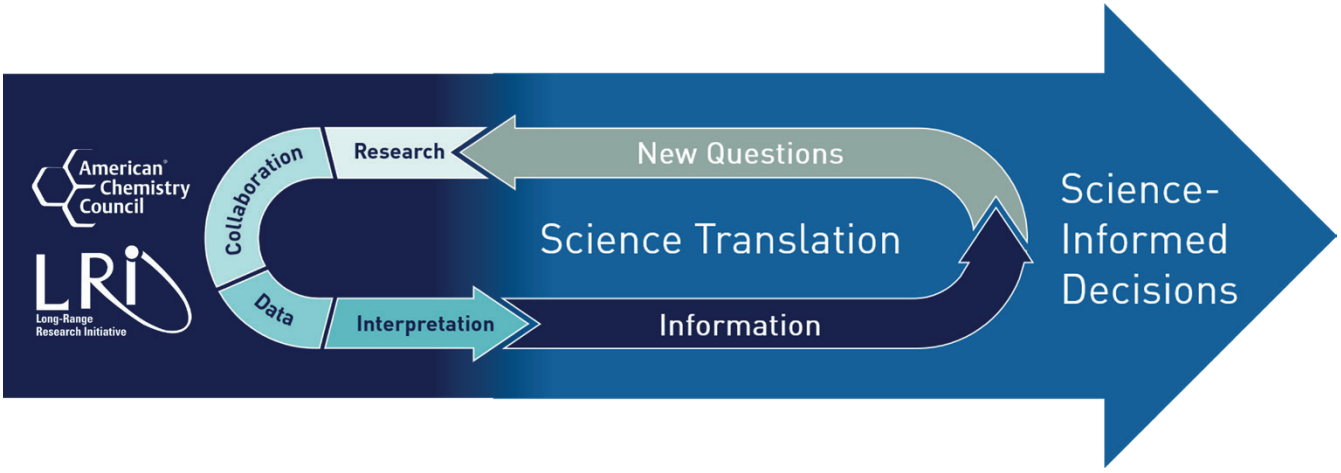
A growing alignment of ethical concerns and awareness of the scientific limitations associated with the use of animals in research and testing, along with recent technological advances, makes the moment ripe to increase ambitions for a faster transition to non-animal science. But current policies and practices are not yet delivering the rate of progress needed.

Countries like Germany, Sweden, the Netherlands and Norway have identified the potential role of policy to drive (scientific) change and have made commitments towards reducing the reliance on animals and transitioning to animal-free science. The



European Union also has the clear objective to fully replace the use of animals in science. However, few initiatives have so far set out clear and comprehensive coordinated strategies, roadmaps or milestones to help achieve the desired goals.

In other areas of societal concern, such as climate emergency, healthcare or education, governments have set out specific aims and ambitions. The European Commission has itself also set out roadmaps with clear milestones where it has made other commitments to achieving a concrete (long term) goal. For example, the EU aims to be climate-neutral by 2050 and to help achieve this goal, the European Commission has put forward a roadmap outlining milestones towards the ultimate target, while identifying policy challenges, investment needs and opportunities in different sectors.

This presentation explores the key elements necessary in a roadmap aimed at accelerating the transition to non-animal science.

Presentation: Oral

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Agrochemical evaluation for the 21st century: Achieving the vision

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A rapidly growing human population and a changing world and climate are increasing the demands on crop production and heightening the needs for safe and effective crop protection products. Although the existing agrochemical safety evaluation paradigm is well established and anchored on classical toxicology methods, it is unlikely to meet the urgent challenge of fulfilling the ever-expanding needs of sustainable agriculture within the desired timetable.

With the science underpinning chemical testing methods is advancing at a remarkable pace, it is critical to consider how to incorporate 21st century approaches into human health and environmental risk assessment decisions for agrochemicals.

To address this issue, a multidisciplinary, international technical science committee comprising participants from government, academia, industry, non-governmental organizations, and other partners was assembled under the umbrella of the Health and Environmental Sciences Institute. This group is proposing a transformative vision for the evaluation of agrochemicals, including a new, fit-for-purpose approach that relies on the integration of state-of-the-art scientific methods, technologies, and data sources, to inform integrated human and environmental risk decisions. Importantly, this approach will also be flexible by design (i.e., based on scientific questions) to adapt to ever evolving local and global needs.

This presentation will provide an overview of this vision, and feature the strategy adopted to design the roadmap that will lead to its realization. Ongoing work, including the exploration of what new approach methods are being used by the agrochemical industry, will be presented as well as how it will inform the development of this new paradigm.

Presentation: Oral

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Real-time lung function measurements of Syrian hamsters for SARS-CoV-2 research

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In preclinical rodent models of respiratory viral infections, disease is frequently evaluated on virological, pathological and surrogate clinical parameters not directly associated with lung function. In humans, however, measurement of lung function is critical for the assessment of respiratory disease severity. To bridge the gap between preclinical and clinical readouts, we set up a whole-body plethysmography (WBP) system in a SARS-CoV-2 Syrian hamster challenge model. While WBP measurements have been investigated extensively for mice and rats in preclinical research, work in hamster models is underdeveloped. To address this, we performed a series of experiments aimed at measuring lung function via WBP in unrestrained SARS-CoV-2-infected hamsters. During our measurements, we obtained highly variable and contradictory values outside the normal physiological range. We hypothesized and confirmed by video surveillance that this was due to background noise created by normal movement/exploration of the hamsters. Importantly, however, we obtained reliable respiratory parameters when hamsters were at rest. We noted that the curiosity of hamsters in exploring the WBP chambers was not substantially reduced even after repeated acclimatization of several hours. We conclude that prolonged, video-supported measurements will be needed to obtain sufficient reliable datapoints during periods when the hamsters are at rest, an approach that is challenging and impractical under BSL-3 conditions. In summary, our experience shows that WBP data obtained from unrestrained Syrian hamsters may require methodic interpretation. We plan to investigate strategies to limit animal movement, which could aid in obtaining more reliable results within a feasible measurement window.

Funded by CEPI.

Presentation: Oral

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Monitoring animal behaviour in infectious disease studies with accelerometers and video

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Automated monitoring of behaviour is a promising method to facilitate reduction and refinement in animal experiments. We explored the use of accelerometers and video to monitor behaviour in infectious disease trials with group-housed animals under biosafety level 2 and 3 conditions. In a recent study, three rams were equipped with two 25 Hz accelerometers, one on the ear and one on the back, and an Aruco-marker for video analysis. Data were collected starting 5 days prior to infection with Toxoplasma gondii through to 21 days post infection. An activity index (AI) was calculated from accelerometer data and time spent drinking was quantified with an automated approach using pixel changes from video. The ear-based AI was higher than the back-based AI, but both showed similar patterns over time. Three days post infection, the AI halved during the daytime/active period, while it slightly increased during the night. These data supported a partial disappearance of circadian rhythm and a general restlessness. Time spent drinking also decreased for each ram. These trends corresponded well to increased body temperatures measured from days 3-9. At 9-11 days post infection, the AI and drinking time returned to pre-infection patterns. As the animal caretakers scored the rams as "inactive" only during days 5-7 post infection, our monitoring provided additional insights into behavioural patterns and disease symptoms. We believe that we can work towards a real-time continuous and automated monitoring system that can facilitate refinement and reduction in experiments with a variety of group-housed species in high-containment conditions.

Presentation: Oral

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Challenges as well as global developments on science and perception of societies

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This presentation constitutes the introduction to the session.

When the first animal testing and marketing bans were put in force, the cosmetics industry had already conducted research and development of alternative approaches for two decades, having founded SCAAT (Steering Committee for Alternatives to Animal Testing) in 1992. The focus was local skin and eye toxicity and mutagenicity. SEURAT-1 as the first public-private partnership in the field of regulatory toxicity had then been conceived to identify principal approaches to chronic systemic toxicity. The following decade has produced remarkable advancement in the identification of new approach methodologies and principles, both by academia and industry, who collaborated in many forms. As a result, the Herculean task of revolutionizing the field of toxicology has now changed from impossible to conceivable. Confidence in Next Generation Risk Assessment (NGRA) is growing, regulatory acceptance increasing. With more than 60% of global consumers opposing animal testing for cosmetics, bans have proliferated to now over 40 countries. Today, cosmetic scientists employ exposure-led and hypothesis driven safety assessment. Many ingredients don't penetrate the skin, allowing their safety assessment based on the Threshold of Toxicological Concern principle. Others can be qualified by using Read Across methods, even first full ab initio assessments become feasible. Here the challenge is to develop and employ approaches that prove safety via the combination of latest systems biology and *in silico* modelling approaches, in order to properly evaluate the complex network of afferent and tolerability-inducing metabolic processes.

Presentation: Oral

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Global harmonisation of acceptance and implementation of 3Rs approaches in biologicals quality control and batch release testing – An industry perspective

Emmanuelle Coppens

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Vaccines and more broadly Biologicals imply stringent quality control testing throughout manufacturing stages to ensure quality, safety and efficacy of each commercialized batch. This testing often entails the use of animals and is performed both by manufacturers and regulatory bodies (National Control Laboratories) in compliance with applicable regulations. Global vaccine manufacturers are confronted to the fact that these regulations are not harmonized worldwide and testing methods for the same product may be region-specific and lead to multiple *in vivo* testing of the same batch.

After presenting what 3Rs approaches currently exist for vaccine quality control and defining a 4th "R" for removal and/or reliance, we will illustrate through some examples the complexity and challenges global companies are faced with.

A focus will then be given on how global harmonization and collaboration leading to better acceptance and implementation of 3Rs approaches can benefit to patients and all stakeholders, what has already been achieved or undertaken and what are the remaining needs.

This work was funded by Sanofi. Emmanuelle Coppens is a Sanofi employee and may hold shares and/or stock options in the company.

Presentation: Oral

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Advancing data interpretation approaches to support the use of transcriptomic points of departure for prioritization and assessment of chemical substances

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A prominent global challenge for regulatory authorities is the continual need to assess numerous chemicals for the hazards and risks that they may pose to humans and the environment, particularly for those with limited data available. New approach methods (NAMs) that provide a high-throughput means of generating data from *in vitro* approaches are being tested as reliable alternatives to traditional assessment methods. The purpose of this investigation was to use gene expression data (i.e., transcriptomics) to identify best practices in data interpretation methods to build confidence in the use of NAM-based approaches in risk assessment activities. A dataset containing 118 diverse chemicals from different cell models and experimental designs was collected. A uniform workflow was applied to compare transcriptomic-derived points of departure (tPODs) generated using different approaches recommended within the literature. tPODs were highly concordant for all but one analysis approach, demonstrating their robustness across datasets and methods. By implementing high-throughput toxicokinetics approaches, tPODs (μM) were translated to human relevant estimates (mg/kg-bw/day), revealing NAM-based estimates to be more conservative (i.e., protective) when compared to traditional PODs for most chemicals. The capacity of tPODs to provide effect levels that are considered protective compared to those derived from animal test methods supports the ongoing transition to applying *in vitro* data for more rapid prioritization and assessment of potentially hazardous chemicals. This study adds to the growing evidence demonstrating the advantages of using NAM-based approaches for hazard assessments in risk assessment applications. This abstract does not necessarily reflect US EPA policy.

Presentation: Oral

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Towards a more efficient chemical safety assessment applying animal-free approaches

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We suggest a long-term objective to revise the current classification system of chemicals to enable an efficient assessment of all chemicals on the market resulting in a higher protection of human health and the environment.

In current discussions, both at EU and international level, there is a concern that not all critical hazards to human health and the environment are sufficiently covered. At the same time, an increasingly complex regulatory system leads to an additional burden of assessment, delaying regulatory actions. The European Environmental Bureau reported that within the EU a harmonised classification of one chemical takes on average 19 years and five months. In addition, the European Environmental Agency (EEA) concluded that approximately 70% of the chemicals on the EU market are not assessed for their safe use.

A re-designed classification scheme should follow the principle of equivalent protection to enable us to make the same decisions, using already classified substances to calibrate the system. The application of NAM-based testing strategies, compatible with the new scheme, should ensure that the 1000s of chemicals currently not assessed are either classified or deemed to be of low concern. The aim is to more efficiently achieve the sustainable use of chemicals based on 21st Century science, without necessarily predicting adverse outcomes.

Presentation: Oral

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Building confidence in the use of new approach methodologies for decision-making: The homosalate case study

Gladys Ouédraogo[1], Matthew Burbank[1], Audrey Noel-Voisin[1], Nicola Hewitt[2], Anne Riu[1], Romain Grall[1], Ann Detroyer[1], Françoise Gautier[1], Sébastien Grégoire[1] and Nazanin Golbamaki[1]

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Although read-across is a well-known data gap filling approach, challenges remain when robust legacy *in vivo* data are not available on analogues. A 10-step framework building on new approach methodologies -NAMS- for using read-across in the context of next generation risk assessment was developed within the Cosmetics Europe Europe's long range science strategy. The applicability of this framework was proof checked by running different case studies, including the homosalate case. This UV-filter has been used for several years in sunscreen products; up to 10% in Europe and 15% in US. The aim is to explore how NAMs could support safety assessment based on read-across, considering the use of homosalate at 15% for face and body products.

After calculating the systemic exposure, a set of NAMs was used following the tiered workflow until risk characterization.

For the exposure, ADME properties were considered and used to translate applied dose into internal concentrations.

Dynamics of parents and metabolites were addressed with *in silico* and *in vitro* tools including, endocrine bioactivity, transcriptomics, *in vitro* pharmacology profiling and cell stress assay.

The margins of safety obtained are protective of human health. This study demonstrated that NAMs can help select and assess analogue similarity and contribute to a quantitative risk assessment. More cases might help build confidence in this tiered approach.

Presentation: Oral

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Tiered approaches for next generation risk assessment (NGRA) of chemicals: Two case studies

Maria T. Baltazar, Paul L. Carmichael, Sarah Hatherell, Predrag Kukic, Sophie Malcomber, Alistair M. Middleton, Iris Muller, Georgia Reynolds, Liz Tulum, Kathryn Wolton and Adam Wood

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NGRA is based on tiered, exposure-led frameworks built on the principles developed by the International Cooperation on Cosmetics Regulation. Published NGRA case studies using coumarin and phenoxyethanol have demonstrated it is possible to integrate exposure estimates and bioactivity points of departure derived from new approach methodologies (NAMs) to make decisions on systemic safety. A systematic evaluation of a first tier NAM toolbox comprising *in vitro* pharmacology profiling, a cell stress panel and high-throughput transcriptomics in multiple 2D cell lines was performed for 10 different chemicals using 24 benchmark exposure scenarios. This showed promise for making consumer safety decisions that are protective but demonstrated that these decisions might be overly conservative given that measures of chemical potency are based on bioactivity, which may not necessarily translate into adverse effects in humans. In this talk, we will include two new case studies, benzophenone-4, a UV filter used in sunscreens and sulforaphane, a component of broccoli, where higher

tier assessments were needed in addition to the first tier described above. For benzophenone-4 the early tiers indicated a high exposure to the kidney mediated via active transport, and therefore specific toxicity renal biomarkers were measured in a primary human proximal tubule model. These case studies have shown that protective and predictive frameworks are not mutually exclusive but are complementary and essential in a tiered approach to safety assessment without animal testing.

Presentation: Oral

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Never say "no": An analysis of corner-cutting measures by IACUCs at major public universities in the United States

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In 1985, the U.S. Congress amended the Animal Welfare Act (AWA) to require that every research facility using AWA-covered animals establish an Institutional Animal Care and Use Committee (IACUC). Yet regulations put forth by the U.S. Department of Agriculture in 1989 to enforce the new amendments allow a proposed animal use protocol to gain approval after being reviewed by only a single member of the IACUC. This process, known as Designated Member Review (DMR), appears at odds with federal law, which reads: "A quorum shall be required for all formal actions of the [IACUC]." The review of protocols is not only a formal action but inarguably a fundamental one for IACUCs. We sought to determine how frequently DMR is used by IACUCs at public universities in the U.S. that receive the most funding from the National Institutes of Health (NIH). Using state public records laws, we acquired documents from 14 universities and compared the usage of DMR to Full Committee Review during the first 6 months of 2016, 2019, and 2022. Our analysis revealed that DMR is overwhelmingly used as the default system for reviewing protocols and modifications to protocols, with several institutions using it more than 80% of the time. This practice is both a symptom and a cause of reduced rigor in the oversight of animal experimentation. We will present our data and discuss the history of DMR, including the possibility that it may be illegal under U.S. federal law.

Presentation: Oral

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Building confidence in NGRA and milestones achieved in terms of acceptance, incl. dialogue with SCCS

Gladys Ouédraogo[1], Catherine Mahony[2], Matthew Dent[3], Andreas Schepky[4], Nicola J. Hewitt[5] and Gerry Kenna[5]

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In the last decades, progress in science and technology has allowed the emergence of next generation risk assessment -NGRA-, especially for the safety assessment of cosmetic ingredients. A joint working group of the International Collaboration on Cosmetics Regulation -ICCR- has developed 9 principles underpinning NGRA. Frameworks recapitulating these principles exist and were used to run case studies demonstrating their used in practice for decision-making. NGRA is exposure led and hypothesis -driven and builds on human biology, with sometimes the use of bespoke tools. It provides more flexibility compared to traditional risk assessment.

The use of NAMs to address local endpoints, skin sensitization and genotoxicity is more and more advanced, and this was translated into OECD defined approaches and IATAs. When it comes to more complex endpoints (such as systemic toxicity and reproductive toxicity), effort is needed to increase the applicability and robustness of NAMs for both safety assessment and address the needs of chemical regulations. Essential steps to address this situation are collaborations with relevant stakeholders globally and along the process of NAM/NGRA's development and evaluation, providing access to the methodologies and approaches via training/education and contract research organizations. This paradigm change calls for the consideration of a new framework for qualifying the fitness-for-purpose of the tools and approaches in a pragmatic way. If some organizations have already embraced this new paradigm, a federated effort is needed for its transformation as a new standard to meet both risk assessment and chemical regulation needs.

Presentation: Oral

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Evaluation of an *in silico* model for predicting pesticide acute oral toxicity

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Acute oral toxicity, based on a dose causing lethality in 50% of animals tested (LD50), is a regulatory data requirement used by the United States Environmental Protection Agency (USEPA) for hazard categorization and environmental risk assessment of pesticide active ingredients (AIs). Oral hazard to humans is based on four USEPA categories ranging from very toxic (LD50 < 50 mg/kg) to practically non-toxic (LD50 > 5,000 mg/kg), while risk to wild mammals is determined by comparing the LD50 to estimated acute oral exposures in the environment after pesticide application. The Collaborative Acute Toxicity Modeling Suite (CATMoS), a QSAR-based *in silico* approach to predicting rat acute oral toxicity, was evaluated as a potential non-animal replacement for the *in vivo* LD50 test. A retrospective analysis compared CATMoS model predictions to the empirical LD50s of 178 previously registered pesticide AIs to determine the accuracy and reliability of the model in the context of the *in vivo* data required and used for pesticide registration. With respect to acute oral hazard categorization, CATMoS successfully predicted EPA toxicity categories III and IV. With respect to risk assessment, the results indicate that CATMoS predictions of 2,000 mg/kg and above are reliable, providing information that could be used as one line of evidence when considering a waiver of the *in vivo* acute oral toxicity test for new pesticide AIs.

Presentation: Oral

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The RepRefRed Society (the Austrian 3R Center) and its impact on animal welfare and quality in science, caring for all. Part I

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Unlike in regulatory areas, in preclinical human and veterinary research the impetus for the transition to animal free research must come from inside the system, from the researchers' perspective.

The path to animal-free research can therefore only be taken by informing the community involved in animal experiments about new tools to strengthen the Refinement as a basis to fulfill the goal of definitive Replacement. On the one hand, this can only work by providing transparent information about the translational problems of individual animal experiments and, on the other hand, by providing tools to Refine individual experiments or jointly develop new animal-free methods.

Therefore, we have revised the classical 3Rs from Russel and Burch and have added new Rs with the goal to develop tools to generate objective solid data and information to help Reduce, Refine and Replace animal experiments.

The theoretical background of this innovative system of the Austrian 3R Center will be presented in this lecture. The practical implementation of our new approach will be shown in Part II.

Presentation: Oral

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Insights from a contract testing laboratory: Adoption of new approach methodologies for adventitious agent testing of biologics

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We present data from our global industry and regulatory insights and experience, as a contract testing laboratory, on the drivers and challenges in the adoption of new approach methodologies (NAMs) for adventitious virus testing.

In the last decade significant advances have been made by regulatory agencies and industry towards the development and adoption of NAMs for the detection of adventitious viruses in biophar-

maceuticals. For example, we have seen the introduction of novel sensitive molecular techniques with broad detection capabilities such as next generation sequencing (NGS) and targeted virus detection techniques such as degenerate polymerase chain reaction (PCR). But have these advances brought about the anticipated rate of replacement in the use of animals for virus safety testing?

We present an overview of NGS and degenerate PCR technologies and look at the drivers and barriers to adoption based on our own experience working with industry and regulators alike. We share data trends on adoption rates, case studies and animal numbers saved during a period of significant regulatory, scientific and 3Rs milestones.

Our data shows that adoption of NAMs is impacted by various barriers but increasingly these are being overcome, in particular as a result of successful regulatory submissions and updated regulatory guidance, as evidenced by the case studies we share.

Adoption of NAMs for adventitious agent testing has faced several hurdles but our data trends, case studies and interaction with industry and regulators, demonstrate how the tide is now turning.

Presentation: Oral

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Centro 3R: Mainstreaming replacement through pervasive 3R education

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The Italian Centro 3R was set up to promote the 3Rs in teaching at universities by creating a collaborative network to share resources and teaching methodologies. When it was setup, a survey was performed to assess the opinions of researchers and students regarding animal experiments and the alternatives and to better identify the knowledge level and needs of students. The survey showed that while Italian researchers are aware of the 3Rs, many of them see animal tests as a ground truth and refer to replacements as complementary models. On the other hand, several science students did not know about the legislation on the protection of animals in scientific experiments or of the concept of the 3Rs. In response to these findings, the Centro 3R's network of universities has established courses and credits focusing on discussions on ethics, one-health, preclinical models, invertebrate models, organ on a chip, *in silico* models to mainstream replacement through pervasive education of the next generation. Although most of the courses are electives, they have tripled in number since 2018 and are heavily subscribed, attesting to the success of the pervasive education strategy.

Presentation: Oral

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Update on the use of new approach methodologies (NAMs) from the US Environmental Protection Agency's Office of Pollution Prevention and Toxics

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EPA's Office of Pollution Prevention and Toxics (OPPT) administers the Toxic Substances Control Act (TSCA) as amended by the 2016 Lautenberg Act. OPPT evaluates the hazard and exposure to new and existing chemicals and is actively applying *in vitro* and computational approaches to support human health and ecological risk assessment. This presentation will showcase on-going research and implementation work. For example, OPPT is implementing a collaborative research program to bring innovative science to the review of new chemicals. This work aims to support major changes in how new chemical reviews can be performed. Advances in data curation and database interoperability, the use of cheminformatics and quantitative structure-activity relationship (QSAR) models, incorporation of a battery of *in vitro* NAMs, and the development of a software tool to integrate multiple traditional and NAM data streams are all components of this collaboration. OPPT is also developing and implementing OECD *in vitro* guideline studies for the evaluation for eye irritation, skin irritation, and skin sensitization in the new chemicals program. Further, OPPT is engaged in developing, updating and applying QSAR models in the evaluation of new and existing chemicals such as EcoSAR and the QSAR toolbox. With regards to advancing ecological risk assessment, OPPT plans to use WebICE and omics testing in fish as part of some existing chemical risk evaluations. As part of the National PFAS Testing Strategy, OPPT has issued test orders on select PFAS and is using a tiered testing approach that includes physical chemical properties and *in vitro* testing.

Presentation: Oral

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Developing an *in silico* virtual cornea for predictive toxicology

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Toxicological outcomes *in vivo* result from complex interactions among molecular, cellular and tissue-level damage responses which are difficult to recapitulate *in vitro* or extrapolate from data-based Machine Learning or molecular-level computer simulations. Mechanistic multiscale, multicellular computer simulations, known as Virtual Tissues (VTs), predict systems-level *in vivo* toxicological outcomes from the detailed molecular and cellular information provided by *in vitro* assays and have been applied successfully by the US EPA in a variety of toxicological contexts (e.g., cleft palate and zonal liver damage on exposure to toxicants). Due to cornea`s experimental accessibility for *in vitro* culture, its relative structural and functional simplicity and its importance in toxicity assays, we have initiated a collaborative effort to develop a Virtual Toxicological Cornea. The simulation, implemented in the open-source CompuCell3D modeling framework, simulates the main cell types in the corneal epithelium and simplified representations of the feedback controlling cell proliferation, differentiation and damage. Subsequent versions will include additional intracellular and intercellular molecular signaling pathways, immune cells, tear film transport and vascular remodeling and transport. Our aim is to successfully recapitulate exposure to a variety of toxicants at different loci and intensities and predict critical outcomes including full recovery, loss of structural integrity and the development of opacity. Developing and validating a VTCornea which correctly predicts structure, function, homeostasis and damage will establish a core set of principles for the development of VTs for other organ systems to complement *in vitro* testing for toxicological assessment.

This abstract does not necessarily reflect USEPA policy.

Presentation: Oral

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Development of a quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) workflow for assessing potential developmental toxicity

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This research effort is focused on the design of a detailed workflow for extending current IVIVE approaches to the pregnant female and fetus and testing the utility of this methodology for assessing developmental toxicity based on *in vitro* bioactivity in a tiered approach paradigm. In the first phase of the project, a comprehensive evaluation of the published literature and publicly available data bases was performed to identify data and models best suited to support development of modified IVIVE equations targeted to maternal and fetal exposures. In this second phase of the project, this information has been used to select chemical case studies for developing and evaluating the utility of the proposed approach. These case studies have focused on a comparison of NAM-based approaches to traditional methodologies (*in vivo* data, PBPK models) to identify specific knowledge gaps. For each chemical case study, the Armitage et al. (2014) model was used to adjust *in vitro* assay nominal concentrations to consider *in vitro* kinetics, followed by QIVIVE using *in vitro* metabolism and PBPK modeling using httk and Berkeley Madonna. Several existing developmental PBPK models were evaluated in the case studies to develop an optimal structure for a generic model. From these evaluations, recommendation for improving the current NAM-based developmental toxicity evaluation approach were identified; most importantly, the need for mode-of-action-specific approaches based on read-across and HTS results.

Presentation: Oral

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How do we get to where we must go in principles of animal ethics?

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For many decades most animal welfare and ethical review groups, with different labels throughout the globe, have used both the 3Rs (replacement, reduction, refinement) and Harm/Benefit Analysis as the cornerstones for welfare and ethical debates and discus-

sions. The gap between these cornerstones can be bridge by still evolving principles of animal ethics. This discussion will present the two main challenges for translational research. The first challenge is tied to the societal benefit to the work, with the second being what is the welfare cost to the animals. From these challenges, principles have been developed, and the proposed principles are detailed, "Principles of Animal Research Ethics" by Beauchamp and DeGrazia. Practical thoughts and possible application of the 3 principles tied to societal benefit – no alternative method, expected net benefit and sufficient value to justify harm – will be presented. Additionally, principles of – no unnecessary harm, meeting the basic needed and upper limits to harm – all part of the welfare cost of a study, will be discussed. While these principles may not fill all the gaps, they do go a way to satisfy many and allow those of varying backgrounds to engage in evidence-based discussions.

Presentation: Oral

405

Three Rs for school-goers – From study to virtual reality

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Benefitting from funding made available under a European Parliament Pilot Project (2018-2020), and a European Parliament Preparatory Action (2021-2023), the European Commission (EURL ECVAM at the JRC) has undertaken initiatives aimed at strengthening Three Rs education for school-goers. Emphasis has been placed on the co-creation, testing and dissemination of Three Rs teaching materials to teachers in order to allow them to deliver Three Rs lessons in the classroom.

Included are ten learning scenarios for primary levels and sixteen for secondary, as well as supporting resources such as references lists, slide decks, games and podcasts. It also provides information on four examples of three Rs careers recorded in podcasts and videos. Last, it is completed with a report that informs key education decision-makers on how to facilitate the incorporation of the Three Rs into their syllabus and curricula.

EURL ECVAM is also developing an open access virtual reality teaching resource to support Three Rs education for students aged 14-19. The goal is to provide an experience which educates on important technologies used in science that do not require animals. This includes the following featured technologies which are used in the EURL ECVAM laboratory: cell culture, cell imaging, microscopy, high throughput screening, *in chemico* screening, Organ-on-Chip, and microelectrode array.

This talk will provide an overview of these resources and explain how they can be used in the classrooms to create both awareness and interest in the three Rs for the young generation. Examples will be presented and discussed.

Presentation: Oral

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Monitoring Three Rs implementation – Open access data for indicator building

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In recent years, many indicators have been developed to measure scientific outputs and innovation using both quantitative and qualitative data. However, only a few attempts have been specifically made to monitor progress on the implementation of the Three Rs.

Recently, EURL ECVAM analysed different approaches for the assessment of the level of development and uptake of alternative methods to animal testing in the European Union through the identification of achieved advancements, impactful trends and future opportunities.

We propose to discuss three examples of open data usage relevant for assessing trends in both animal and non-animal experimentation in domains such as basic and applied research:

The EU statistics on animal used for scientific purposes showing detailed information of all uses of animals, including the purpose, the genetic status of the animal, and the actual severity as a result of the scientific procedure.

The EU non-technical project summaries of authorised projects providing information on where, why and how animals are still being used, going beyond the numbers for a better understanding of different scientific use areas and why animals cannot yet be fully replaced in these areas.

The open data resources allowing bibliometric analysis through the retrieval of information on the number of scientific publications based on animal or non-animal models, factoring in geographical location, biomedical research areas and publication types.

We will present some of the indicators we built (trends in animal uses, projects information, evolution of animal versus non-animal publications), highlighting the information that they bring and their limitations.

Presentation: Oral

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Novel approach methodologies and change management: A need for a professional Master program

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Rationale: An increasing number of new techniques is available to replace existing animal experiments. All these innovations are contributing to a tremendous acceleration in 3R-capabilities within the animal research community and require adjustments, e.g., from regulatory agencies. However, this transition faces difficulties regarding translatability, reproducibility and reliability between data obtained from animal and non-animal models. This requires a new professional who can cope with these challenges on substantive scientific, technical and communicative grounds.

Aim: The HU University of Applied Sciences Utrecht and the Utrecht University are developing a practically-oriented master's degree in New Approach Methodologies and Change Management. The target group of the programme is the professional in the field. Their field of expertise and critical view is fundamental to successfully completing the master's degree. The students obtain their degree through continuous education via, for example, challenged based learning on realistic and authentic assignments from the field. The program will be interdisciplinary, integrating professionals from different fields of biology, bioinformatics and social sciences.

Professional role: The graduated master will function as a connecting link between the *in vivo* scientific community and the non-animal testing community. The professional role could be practising as a hands-on laboratory technologist or as a change manager at a research centre, company, animal facility or governmental authority. Thereby, this new professional can help catalysing the transition to, and the acceptance of, innovative animal(free) testing methodologies.

Presentation: Oral

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A novel *in vitro* liver culture device for continuous bile recovery

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Understanding the excretion pathway of drugs in the liver, especially biliary clearance, is crucial in drug development. Metabolites excreted via bile are partly involved in enterohepatic circulation which makes it difficult to predict the excretion of metabolites out of the body. In addition, some drugs excreted into bile can cause liver cholestasis by influencing the excretion of bile acids via the inhibition of transporters.

In *in vitro* models, sandwich-cultured hepatocytes have been the golden standard for assessing bile (Xingrong Liu et al., 1999). However, since bile cannot be evacuated in such a culture system, bile has to be recovered from hepatocytes in an invasive manner, which causes the collected bile to be diluted excessively within the collection buffer, hampering an accurate measurement of biliary metabolites.

In this study, we aim at developing a novel *in vitro* hepatocyte culture system which enables continuous bile recovery by combining microfabrication techniques and coating proteins for bile canaliculi formation (Yue Zhang et al., 2020). We fabricated a device that consists of a polydimethylsiloxane (PDMS) disk with radial microchannels (width: 3 µm, height: 5 µm) and a PDMS tube at its center, serving as a bile collection port connected with the microchannels. Furthermore, fluorescein-labeled bile acid excreted by hepatocytes cultured on the device was successfully recovered from the bile collection port.

This study is expected to revolutionize the method of bile analysis, which has not been technically improved for more than 20 years, and to enable accurate evaluation of pharmacokinetics and drug-induced cholestasis.

Presentation: Oral

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Status of quantifying adverse outcome pathways to support next generation risk assessment

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Quantification of adverse outcome pathways, the development of qAOPs, involves developing or applying models to the relationships between molecular initiating events and/or key events. The current status of qAOPs to support Next Generation Risk Assessment (NGRA) has been evaluated. At the current time there are relatively few qAOPs that have practical use in NGRA. There is a need to develop case studies to illustrate their use and investigate the breadth of modelling techniques that may be applied. Within the European Union RISK-HUNT3R project, part of the ASPIS Cluster, qAOPs are being developed for a number of adverse outcomes. The development of qAOPs is driven by three distinct factors, namely the availability of a robust AOP, sourcing relevant data and the requirement for a model to fit the data including an assessment of uncertainties. Once developed, the qAOPs provide a means to use New Approach Methodology (NAM) data to learn more about downstream effects in the AOP. Their use for regulatory risk assessment Is very much in its infancy, however, it is anticipated that qAOPs will contribute to the weight-of-evidence to make a decision.

This work has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 964537 (RISK-HUNT3R), which is part of the ASPIS cluster.

Presentation: Oral

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Policy change underpinned by science: Accelerating a shift towards human-focused research and testing by working with policy makers

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The evidence supporting a transition in biomedical science from animal-oriented research to humane, advanced human-focused methods (including New Approach Methodologies (NAMs) is formidable. The resulting pace of change, however, even in light of powerful ethical and scientific rationales, has been slow. It seems clear that one crucial area for the efforts of organisations seeking to expedite this change, in addition to using scientific and ethical cases to convince researchers, industries and the public to support and elicit change, is in policy change via public affairs/political work underpinned by science. In the UK, we are making strides and achieving encouraging results with our work aimed at achieving policy changes to drive a shift towards human-focused science. We will discuss our multi-pronged actions to raise awareness of this issue among policy makers, in order to alter their mindset and enable changes in the law to accelerate a scientific paradigm shift that will benefit animals and humans. These include highlighting scientific, animal-welfare, human medical and economic benefits, with specific examples to clearly highlight success stories and to illustrate the way forward in other areas of biomedical research and testing. We outline specific policy requests that we believe will amplify and broaden these successes, and how we believe we can make our voice stronger than it has ever been by working with multiple and diverse stakeholders. Available evidence shows that political efforts are now working in many countries, and that what we are doing to achieve change in the UK could be helpful internationally.

Presentation: Oral

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United States Animal Research Openness Initiative

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This presentation will introduce the premise and progress of the United States Animal Research Openness (USARO) Initiative. As a community we feel that increased openness will foster meaningful public conversations about the critical role of animals in research, teaching and testing. In order to accomplish this goal, the Initiative reached out to organizations across the United States that rely on the responsible use of animals in research.

Globally there are at least 9 countries that have formal animal research openness agreements. These efforts are resulting in a better understanding of when animal models are necessary. The USARO Initiative was first discussed in 2018 and since has grown into a formal process with a proposed agreement that consists of over 100 individual representatives from organizations across the United States. USARO is focused on bringing the biomedical research community together to increase communications by institutions to the public about how, when and why animal research is necessary. Openness communication will involve being clear

about when animal models are used and when non-animal models are used, as well as the important contribution that each makes to the continued understanding of science and the development of safe and efficacious therapies.

USARO consists of several committees to move this effort forward, including those focused on developing the strategic plan, writing a one-pager document and other supporting resources, designing and distributing an openness survey, recruiting exemplars and ambassadors, and assuring effective communications. We will share the progress and the information that is being made publicly available.

Presentation: Oral

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Compassion resilience for working with research animals

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This presentation is focused on providing an overview of a Compassion Resiliency program at a large institution. Personnel that interact with research animals can form strong bonds with the animals and it is essential that organizations provide resources to help individuals process what they are feeling and prepare for any potential challenges.

Compassion resiliency is about having the resources to deal with feelings that can easily overwhelm an individual. Professional quality of life is a measurement of the quality of "work life" and consists of both the positive feelings about our work, compassion satisfaction, and the negative feelings, compassion fatigue, which can include burnout and secondary traumatic stress. In order to build resilience, it is important for an individual to have the resources to help them identify how to best support themselves and others. It is essential that a resiliency program includes resources at both the individual and organizational level.

The University of Washington has had an active Dare 2 Care (D2C) Program since 2016 that is focused on compassion in science. This program is designed to provide individuals with tools to proactively prepare for potential adverse feelings and to provide support when something happens that they weren't expecting. The program has evolved over time, and we would like to share what we have learned and how we have been able to adapt to the changing needs of our community. This will be an interactive session with options to choose what scenarios the audience would like to discuss.

Presentation: Oral

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Unified ethical principles and utilization of the Basel Declaration

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The purpose of this presentation is to propose a common framework for international researchers to rely on when they require animal models to support their scientific discoveries. The rationale for providing this guidance is to foster global collaborations with confidence of a commitment to a foundational set of principles for assessment of their work.

We have previously done an analysis of the 3Rs and proposed a unified ethical framework to support international collaborations and the standardization of ethical principles regarding the use of animal models. This approach combines the common guiding principles that are relied on for clarifying when the use of animal models is necessary and the thoughtful approach to this consideration. These principles include the 3Rs: Reduction, Refinement and Replacement; the 3Ss: Good Science, Good Sense and Good Sensibilities; 3Vs: Construct, Internal and External Validity; the 4 Fs: Fundamental Principles of the necessity of biomedical and animal research and minimization of pain and distress, and the 6 Principles that encompass a harm-benefit and minimization of harm premise.

We suggested a preamble to this would be a more formal use of the Basel Declaration which clearly states a commitment to science and animal welfare. In this presentation we will cover the important role of individual accountability and commitment that can be attested to by acknowledging the Basel Declaration. This proposal will provide suggestions for future incorporation on how the declaration can be integrated into one's work and assist with an open dialog on the use of animals in research.

Presentation: Oral

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Ambition statement on innovation in higher education using fewer laboratory animals in the Netherlands

Daniela Salvatori[1] and Ambition statement innovation in higher education using fewer laboratory animals[2]

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Initiated by the Dutch Government in 2018, the Ministry of Agriculture, Nature and Food Quality has been managing an alliance aimed at accelerating the transition to animal-free innovation. In 2019, the Dutch Universities and Medical Centra joined and wrote an ambition statement aimed at using fewer laboratory animals in Dutch higher education.

Annually, a total of approximately 9,000 animal experiments are performed in bachelor, master and postgraduate education in The Netherlands. The working-group formulated the following recommendations:

- In the Bio-Medical Studies Bachelor's and Master's programmes, if the same learning goals can be achieved using animal-free methods, it is mandatory to implement animal-free methods.
- In bio-veterinary programmes, a step-by-step approach based on animal-free methods before approaching the live animal must be implemented and supported by animal-free skills labs (models and simulators) and earlier clinical teaching on real patients.
- Most animals are used in the Laboratory Animal Science (LAS) courses for practical training of those who actually perform animal experimentation themselves. It is time to modernize the LAS courses by expanding the theoretical part and including animal-free innovations proposing a new format that does not use live laboratory animals at all.

It appears that where traditional animal use persists, this is most often due to uncertainty about the educational efficacy of humane alternatives and a lack of awareness of existing resources. The outcome of animal-free teaching models should focus on learning goals and benefits and should no longer be measured against the animal-based method as the traditional gold standard.

Presentation: Oral

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Developing and embedding education programmes for the transition towards non-animal methods at Utrecht University

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The Dutch government has established a Transition Programme for Innovation without the use of animals (TPI). Utrecht University, the University Medical Centre Utrecht and the University of Applied Sciences Utrecht have joined forces in TPI Utrecht to effectively support and further boost the transition. TPI Utrecht strives for excellence in science and education. One of the core elements of our TPI strategy is to create educational programmes for students, professionals and general public. Students are key stakeholders and "effective change agents", but their perspective remains underrepresented. Our studies explored students' viewpoints focusing on their beliefs, values, and the motivations of students to join TPI-inspired courses. We show that students share the ethical and scientific values that inspire the transition, and that their reflections on the socio-political landscape provide valuable insights on current and future challenges. TPI Utrecht is working towards development and embedding of animal-free innovations in biomedical and veterinary studies while maintaining a high quality of education. Education programmes within the life science domain offer structured animal-based courses at the under and postgraduate levels, while such courses are more limited for non-animal methods. TPI Utrecht is developing extensive education programmes that serves as a basis for our educational ambitions. This includes education with professionals and students and on-demand for specific groups of professionals from industry and academia. TPI Utrecht is experimenting with new ways of interdisciplinary and active learning such as challenge-based learning courses aimed at developing critical skills in this subject.

Presentation: Oral

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Lab on a laptop: Beyond the experimental model

Ermes Botte[1,2,3], Piera Mancini[1,2], Flavio Fontanta[1,2], Chiara Magliaro[1,2,3] and Arti Ahluwalia[1,2,3]

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Although tissue and organs obey physical laws, they are characterized by intrinsic complexity and variability that are very difficult to model experimentally. More importantly, uncoupling aspects related to differences in protocols and experimental conditions from those arising from variability intrinsic to nature is well-nigh impossible. *In-silico* models can provide crucial support to *in-vitro* experiments as they allow the high-throughput and comparatively low-cost generation and study of virtual tissue constructs; for instance, variables and conditions can be easily tuned and parametrized. We have developed three dimensional models of spheroid and organoid morphology which account for thermodynamic, evolutionary, and scaling laws and include environmental fluctuations (different media conditions) as well as intrinsic variability arising from thermal noise and measured variations in cell and construct size. The models are based on the integration of genetic algorithms, statistical mechanics, allometric scaling and finite element simulations of individual and collective cellular resource (mainly oxygen) consumption. We show that biological variability impacts the allometric scaling behaviour of the cellular constructs and that their morphology and size is sensitive to changes in nutrient supply, which is dependent on media height (for oxygen) and volume (for glucose and other soluble molecules). The predictivity of models based on a limited number of physical and metabolic laws suggests that the progressive improvement of integrated computational models could, in the future, augment or even substitute *in vitro* models providing a more sustainable, high-fidelity way of conducting experiments, particularly if our computers and laptops are powered by renewable energy sources.

Presentation: Oral

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Beyond serum-free cell culture media design: A systematic approach towards long-term animal-origin-free cultivation of RTgill-W1

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Based on its pioneering role in standardized toxicity testing with fish cells (ISO standard 21115 and OECD TG249), the RTgill-W1 cell line has been chosen as a "proof-of-concept" model for developing a successful strategy for fetal bovine serum (FBS) replacement. Even though the standardized tests using the RTgill-W1 cell line are devoid of serum, serum remains an important component for routine fish cell growth.

A high-throughput 96-well plate RTgill-W1 cell proliferation assay was established to enable systematic screening of media components, individually and in mixture. The design of the experiment corroborated the selection of the final culture media combinations that yielded the best metabolic activity, cell number, and cell morphology of RTgill-W1 cells. Finally, the most promising mixture was tested for long-term cell proliferation by applying two adaptation strategies in parallel: direct ("sink-or-swim" cells) or sequential adaptation by gradual dilution of FBS with the serum-free medium ("weaned" cells). The RTgill-W1 cell line was successfully adapted to serum-free media using both procedures. Yet, complete adaptation occurred more quickly in "weaned" cells than in "sink-or-swim" cells: the former achieved the benchmark passages of 3 and 10 more quickly than the latter. RTgill-W1 serum-free cells have been passaged more than 30 times, during which cryopreservation, cell doubling time and functionality assessment in OECD TG249 has been successfully tested.

This is the first report of a serum-free medium for long-term culture of a fish cell line. We now use this as a base to further progress toward an entire animal-origin-free fish cell culture medium.

Presentation: Oral

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The national 3R centers and their impact on animal welfare and quality in science part 2 – Closing the gap of refinement

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Unlike in regulatory areas, in preclinical human and veterinary research the impetus for the transition to animal free research must come from inside the system, from the researchers' perspective.

The path to animal-free research can therefore only be taken by informing researchers and all the employees involved in animal experiments about new tools to strengthen the Refinement of animal experiments with the goal of a definitive Replacement. On the one hand, this can only work by providing transparent information about the translational problems of individual animal experiments and, on the other hand, by providing tools to Refine individual experiments or jointly develop new animal-free methods.

The Austrian 3R Society has therefore developed tools to provide researchers and all those involved in animal research with objective data to help Reduce, Refine and Replace animal experiments.

The goal of the project is defined in providing solid data and information.

By improving the old 3Rs from Russel and Burch and by addition of several new Rs we show an easier, new approach to ultimately reduce the number of animals in animal experiments and improve the quality of life of the Laboratory animals.

Presentation: Oral

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Refining and removing global mammalian acute toxicity testing requirements

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Acute toxicity tests are currently conducted as part of global regulatory risk assessment and hazard classification packages for industrial chemicals and agrochemicals. Historically, the aim of acute toxicity tests has been to determine the dose or concentration which is lethal to 50% of the animals treated. They are therefore associated with suffering in the test animals, and there is great scope to apply the 3Rs in this area of testing. This presentation will describe previous data-driven collaborative efforts that provide scientific evidence to support a) the refinement of mammalian acute toxicity tests for (agro)chemical testing (via oral and inhalation routes) through the assessment of evident toxicity, and b) the removal of the requirement for acute toxicity testing of pharmaceuticals. It will also highlight areas where there is potential to further move away from traditional regulatory acute toxicity tests towards more predictive methods, for example through the application of computational approaches. Ultimately, global harmonisation in the acceptance of new approaches will be required before the maximum 3Rs impact can be achieved and the requirement for acute toxicity tests be eliminated altogether.

Presentation: Oral

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Application of *in vitro* transcriptomics point of departure and IVIVE in early agrochemical discovery programs

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Recent efforts identifying alternatives to animal testing have focused on predictive and precision testing, with ample development of high-throughput screening, new technologies, and alternatives to animal testing. In particular, transcriptome-based *in vitro* approaches could provide a powerful tool for regulatory decision-making among with physiologically based kinetic (PBK) modelling that represents a well-established and accepted methodology for improving quantitative *in vitro*-to-*in vivo* extrapolation (IVIVE). Furthermore, while high-throughput *in vitro* toxicity screening provides an efficient way to identify potential biological targets, reliance on the nominal chemical concentrations in these *in vitro* assays as an indicator of bioactivity may misrepresent potential *in vivo* effects due to differences in clearance, protein binding, bioavailability, and other pharmacokinetic factors. PBK modeling provides an effective framework for conducting quantitative IVIVE to solve this dilemma. Given that previous findings suggested apical and transcriptomic endpoints show similar points of departure (POD) *in vivo*, we tested the hypothesis that an *in vivo* transcriptional POD could be accurately predicted from an *in vitro* POD using PBK modeling. Transcriptional profiles from rat liver samples and rat primary hepatocytes exposed to different chemicals from the TG-GATEs database were used to generate transcriptome PODs followed by PBK based IVIVE to estimate rat-relevant exposure. Our findings suggest a stronger correlation between *in vitro* and *in vivo* transcriptional PODs using measured plasma

protein binding and hepatic clearance data when data were generated using appropriate *in vivo* dose selections. These findings suggest that PBK modeling improves the prediction of *in vivo* PODs from cellular-based transcriptome data.

Presentation: Oral

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The Beyond Animal Testing Index (BATI): A benchmarking tool for a world beyond animal testing

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The transition to animal free innovation is high on the political agenda inside and outside the European Union. While the original definition of "Replacement" focuses on the replacement or avoidance of the use of animals in science, the contemporary definition includes accelerating the development and use of predictive and robust models based on the latest science and technologies, to address scientific questions without animals. The Beyond Animal Testing Index (BATI) is, to our knowledge, the only benchmarking instrument designed to provide insight into the activities and contribution to the transition to animal free innovation by research institutes. The BATI allows participating organisations to learn from each other and stimulates continuous improvement.

The BATI was modelled after the Access to Medicine Index, which benchmarks pharmaceutical companies on their efforts to make medicines available in developing countries. A prototype of the BATI was tested with five academic organisations. The test demonstrated the usability and effectiveness of the BATI as a benchmarking tool. Analyses were performed across five different domains and organisations were assessed for their commitment, transparency and performance based on existing reports and interviews. The participating institutes concluded that the BATI served as an internal and external stimulus to share, learn and improve institutional strategies towards the transition to animal free innovation. The BATI also identified gaps in the development and implementation of 3R technologies. Hence, the BATI is a suitable instrument for monitoring the effectiveness of policies and is ready to be used for future benchmarking at a larger scale.

Presentation: Oral

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Secondary pharmacology: Strategies, deployment, and impact on the safety of new medicines

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Adverse events (AEs) often occur as a result of off-target or secondary pharmacology (2P) activities. Consequently, *in vitro* profiling of new chemical entities (NCEs) across a range of protein targets (e.g., GPCRs, ion channels, transporters, enzymes including kinases) with known association to AEs is used in pharmaceutical R&D. As part of the IQ DruSafe consortium, a 2P working group was established, comprising representatives from 18 mid to large size pharmaceutical companies, to conduct a review of 2P strategies and share experiences, and to propose recommendations for best practices and future application for the industry. A detailed database capturing an overview of the strategic, scientific, and operational aspects of 2P screening was created. Despite a diversity of screening strategies and target panels among the companies surveyed, there has been broad adoption of screening against certain targets. An additional set of bespoke or uncommon targets was identified that should be considered from a safety perspective. Moreover, extending the diversity of target classes screened to include a set of safety-related kinases, as well as potentially enzyme and epigenetic classes should be considered. Across-the-board adoption of 2P screening has coincided with more selective NCEs reaching the market in the last decade, and a decreased incidence of AEs for newly marketed drugs, demonstrating a positive impact of 2P on drug safety. Such an approach improves effectiveness and efficiency by identifying high quality drug candidates with higher likelihood of success, ultimately contributing to reduced animal experimentation.

Presentation: Oral

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Nonanimal research in the news: Capturing attention, making waves

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Working with the news media and social media is an essential part of a complete approach to bringing the 3Rs to the public. These communications strategies can elevate awareness of the advantages of nonanimal methods, the needs for regulatory acceptance, and the importance of next-gen education. Public awareness (which can lead to public support) is a major factor that can accelerate changes in scientific practice and policy. This presentation will discuss a variety of approaches for researchers, advocates, and communications teams to use the media to amplify their impact for patients and animals. Working with the news media requires an understanding of journalists' and news outlets' needs, limitations, and strengths; an understanding of the public's appetite for different topics and stories; the ability to discern how to convey evidence from research effectively and appropriately; and the ability to provide the broader context and significance of research findings. Publishing to blogs and social media directly can allow for a more detailed and visual expression of research findings and implications and can be tailored for a more niche audience. Overcoming the platform-specific restraints of social media (i.e., character limits on Twitter or video dimensions on TikTok) requires innovation that can sometimes cultivate more creative expression. Using these means to communicate the advantages and successes of nonanimal research to the public and stakeholders can have a big impact, and giving communications strategies adequate forethought can increase chances of moving the needle toward a more ethical and effective research enterprise.

Presentation: Oral

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Putting patients first: A human-centered approach to translating *in vitro* virology research into clinical practice

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The COVID-19 pandemic has highlighted the critical need for effective and efficient translation of *in vitro* research into clinical practice. Although there has been remarkable progress in understanding the virology of SARS-CoV-2, the causative agent of COVID-19, the translation of this knowledge into effective clinical interventions has been challenging.

The poor translation of *in vitro* virology research into clinical practice can also be attributed to the limitations of animal models. While animal models can provide valuable insights into the pathophysiology of viral infections and the efficacy of potential treatments, they may not fully reflect the complexity and heterogeneity of the human disease. For instance, animal models of COVID-19 have been criticized for not fully replicating the range of symptoms and disease severity seen in humans. This can lead to a lack of generalizability of research findings and limit the effectiveness of interventions in human populations.

Here we present an interdisciplinary implementation that identifies, examines, and validates human mechanisms of SARS-CoV-2 infection, resulting in improved translation of *in vitro* virology research into clinical practice. In a double-blinded clinical study conducted on 38 severe-hospitalized COVID-19 patients, we are able to show improvement in viral load, inflammation, and immune markers, as well as hospitalization duration and withdrawal from supplementary oxygen. This framework can serve as a roadmap for developing effective and equitable interventions that can improve the health and well-being of patients affected by COVID-19 and other infectious diseases.

Presentation: Oral

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High-throughput preclinical model of breathing human alveoli

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Bioengineered microphysiological models that can recapitulate the tissue structure, cellular organization, and mechanical dilation of the human lung are becoming increasingly important for advancing our understanding of pulmonary pathophysiology and improving the translation of preclinical drug testing. Airflow and mechanical actuation are the most unique features of alveoli and have profound effects on tissue maturation and disease progression. Significant advances have been made in applying mechanical stretching to microphysiological models, but existing systems have very limited experimental throughputs and are not compatible with large-scale drug screening. We developed a high-throughput bioengineered breathing alveoli model in a customized 384-well-plate where a large array of tissues can be simultaneously cultured

and mechanically actuated. We 3D-printed a sacrificial bioink to pattern an alveoli-like structure inside a lung fibroblast-embedded fibrin gel. The bioink was then removed, leaving behind an alveolar cavity that could be populated with primary alveolar epithelial cells. To emulate breathing, we connected the platform with an external ventilation system that can introduce oscillating air pressure to the entire plate and mechanically actuate up to 64 tissues simultaneously. Using this platform, we aimed to study the effects of mechanical actuation on alveolar cell maturation, gene expression, and cytokine secretion. Furthermore, we sought to understand the effects of breathing-induced mechanical forces on TGF-β1-induced fibroblast activation which leads to pulmonary fibrosis. In summary, this breathing alveoli model will reveal the importance of mechanical actuation in pulmonary disease progression and provide a more predictive preclinical model that is compatible with high-throughput drug screening and development.

Presentation: Oral

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Mapping of DNT NAMs' signaling pathways in human physiology and disease

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The current regulatory DNT guidelines are not fit-for-purpose for the hazard assessment of the immense chemical universe. They require using *in vivo* tests that are burdened with ethical, practical (time & cost), and scientific (limited predictivity for human health) issues. Thus, more reliable and efficient human-based NAMs are needed. Their extensive characterization including data reliability and human relevance is crucial to reduce their uncertainty and increase regulatory confidence.

Thus, our goal was to elucidate the biological applicability domain of the DNT *in vitro* NAMs, used in the ONTOX project, by investigating 19 signaling pathways known in human physiology and disease. A second goal was to create a physiological map offering an overview of human brain development with integrated experimental results of the detected signaling pathways. The signaling pathways modulatable in the assays were experimentally determined by their inhibition and/or activation. The outcomes were discussed in relation to the known implications of these pathways in human neurodevelopmental diseases and critically compared with findings in rodents.

In conclusion, the presented results will ultimately lead to support the regulatory acceptance of the DNT *in vitro* NAMs by increasing the understanding of their biological applicability domain. Furthermore, the mapped results in the physiological map will be an integral part of a new innovative strategy composed of an AI-driven and ontology-based combination of *in vitro* and *in silico* NAMs able to predict the effects of chemicals on the developing brain, developed in the framework of the ONTOX project.

Presentation: Oral

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Refinement in fracture management of the hindlimb post tibial fracture: Establishment of a walking cast for sheep (SWC)

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Casting after fracture treatment of the limbs is standard treatment in both human and veterinary medicine. Despite sheep are one of the most relevant species in the testing of new materials for osteosynthesis using tibia defect or fracture models, to date, there is no system for cast stabilization ensuring anatomically correct position and function of the hindlimb during healing process. The actual gold old standard is the usage of sling suspension for the animals for 4-6 weeks after surgery. During this period, the animals can barely move around via a ceiling attachment but cannot show any species-typical behavior (lying down, standing up) and group housing is also unfeasible.

In our study, a walking cast for sheep (SWC) was developed, which enables the animals to move in a species-typical manner directly after surgery. 9 Rhone-race sheep (female 65 kg ± 8 kg BW) were observed for 4 weeks after osteosynthesis post tibia defect (mechanical defect drilling) and cast attachment. All animals began weight bearing on the operated leg from the first postoperative day during the walking process with a lameness-scale 1 (scale 1-4) during the first postoperative week. The determined individual single step length was very uniform with 76.9 ± 0.89 cm. Group housing was performed from the third day after surgery on. Weekly radiographic control and cast change ensured the correct fitting

of the cast and healing process. This study shows the implementation and the exemplary usability of the walking cast in sheep as refinement up to a weight category of 70 kg bodyweight.

Presentation: Oral

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Towards regulatory acceptance of qAOP-based NGRA through integration with a qualified open-source PBK framework

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The integration of PBK models with complex mechanism-based quantitative adverse outcome pathway (qAOP) systems-biology models leads to an increase in model complexity and number of use-scenarios, which regulatory bodies demand to be qualified. This becomes challenging when changes in the software platform occur, requiring new features for efficient model maintenance and continuous (re-)qualification. The Open Systems Pharmacology Suite (OSPS; www.open-systems-pharmacology.org) is a robust, reliable, and user-friendly open-source software for life-sciences applications. Designed with a modular concept and optimized for PBK modeling of small and large molecules in different species and populations and for integrating cellular-level systems-biology, it offers efficient, flexible, and transparent multi-scale systems toxicology modeling and simulation.

In OnTOX, recent software developments for the OSPS that address these challenges in PBK and complex systems modeling will be adopted. These include

1) an automated qualification framework to ensure continuous platform and model qualification
2) a model exchange/plug-and-play interface for robust management, modification, and extension using existing standards such as SBML.

In OnTOX, data from various biological levels will be integrated, improving efficiency in data analysis across biological scales and model applications which will be outlined on the specific use-cases.

The integrated modular modeling concept will allow continuous implementation, qualification, and deployment of complex systems models and use-scenarios, leading to increased adoption of computational models for NGRA and NAMs compliant with (future) regulatory requirements.

Presentation: Oral

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Quantitative *in vitro-in vivo* extrapolation (QIVIVE) in chemical risk assessment

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In recent years, there has been a growing trend toward applying *in vitro* methodologies in chemical safety assessment. However, to assess the potential risks these chemicals may pose to human health, it is crucial to interpret these findings in the context of whole living organisms. Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) is a vital tool in achieving this goal. QIVIVE refers to the translation of *in vitro* (i.e., in the test tube or cell culture) concentration-response relationships to *in vivo* (i.e., in living organisms) dose-response relationships in humans and animals.

Several QIVIVE approaches have been developed and are valid for screening and prioritizing chemicals and determining equivalent effect doses. Physiologically based kinetic (PBK) models, which simulate the fate of substances in body tissues, are essential for extrapolating *in vitro* effect concentrations to *in vivo* bioequivalent exposures. In the future, it will be necessary to democratize and standardize PBK modeling approaches to ensure consistency and acceptance from regulatory agencies. This presentation will introduce concepts of the QIVIVE approach and recent experience on how QIVIVE can be applied in chemical risk assessment by illustrating current research efforts from private and international projects. The presentation will also discuss recommendations from the 2021 QIVIVE ECETOC expert workshop and the OECD DNT IVB QIVIVE addendum to increase the potential for timely acceptance of a more progressive and tailored QIVIVE approach from the regulatory community.

Presentation: Oral

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Creating community and opportunity for early-career researchers

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Students and early-career researchers (ECRs) who aspire to work with animal-free, human-relevant New Approach Methodologies (NAMs) generally face additional career challenges compared to those who are working within the current animal-use paradigm. Fortunately, several organizations have recognized the importance of supporting ECRs to use NAMs from the start of their careers. Through immersive programs, workshops, funding, and research opportunities these organizations are committed to train the next generation of scientists to work with animal-free, human-relevant NAMs and, thus, to help accelerate advancements in biomedical and translational research. This presentation will highlight international opportunities for ECRs including programs offered by the Johns Hopkins Center for Alternatives to Animal Testing (CAAT), the Animal Protection Commissioner of Berlin, the Physicians Committee's Early-Career Researchers Advancing 21st-Century Science (ERA21), Animal Free Research UK, and more. These programs, among others, are working to speed up progress in ethical and effective scientific research by creating a new generation of scientists who utilizes and champions nonanimal research methods. Furthermore, these programs foster networking opportunities to help ECRs strengthen their connections, build confidence, and gain fresh perspectives. If we are to meet the challenges and opportunities of the next generation of biomedical investigators, the vision, creativity, commitment, and hard work of the research community must now be focused on restructuring our approach to training.

Presentation: Oral

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Using immortalised blood cells to study the long-term effects of common respiratory viruses

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Chronic respiratory illnesses affect more than 500 million people worldwide and are a leading cause of death. They are commonly caused by viral infections such as influenza, enteroviruses and coronaviruses including SARS-CoV-2. Infections cause damage to lung epithelia and can lead to complications such as pneumonia and acute respiratory distress syndrome. Additionally, many viral infections cause longer-term effects including the post-viral fatigue illnesses Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Long COVID. Both multisystem disorders have overlapping symptoms including debilitating fatigue, exertion intolerance, post-exertional malaise and neurological dysfunctions to name a few. There are no diagnostic tests, limited treatment options, and patients are often told it is psychological. With millions affected worldwide the social and economic burden is substantial, therefore we urgently need insight into the disease pathophysiology to develop diagnostic and treatment strategies. Here, we study the molecular mechanisms underlying these illnesses and aim to identify potential therapeutic targets and biomarkers. We use the most accessible tissue, blood, isolated from afflicted individuals and healthy controls. Blood cells however are not metabolically active nor proliferative so defects in metabolism are difficult to detect. To overcome this, we immortalise the blood cells to create lymphoblasts. Lymphoblasts have been used extensively to study many disorders. Here, we have used them to perform functional assays and employed various 'omic strategies to uncover defects in the way these patient cells make energy, and identified key differences in the expression of genes which can accurately distinguish disease from control and could be developed into diagnostic tests.

Presentation: Oral

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Beyond harm-benefit – Demanding a life worth living for laboratory animals

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The order by which the 3Rs were originally proposed clearly reflects a value hierarchy: firstly, replace, then reduce, only then refine, when all Replacement and Reduction options are exhausted. Such view stems from the notion that animal research is inescapably harmful. It is nevertheless seen by most as ethically acceptable if no alternatives are available, animals are "respected", and harms to them can be outweighed by progress in human/animal health and safety. However, such benefits might be hard to predict or quantify. Moreover, laboratory animals neither partake in research voluntarily, nor is it usually carried out to their benefit. It is therefore worth pondering under what circumstances benefits to the animals being used themselves could offset the harms they endure. Indeed, accepting animal research only if the animals involved are allowed a good life, or at least a life worth living, would ease the pressing dilemma it brings forth, particularly when no direct benefits can be ascertained. But can we provide laboratory animals good lives? Considering they are kept under controlled environmental conditions, free from natural predators or scarcity, and euthanized by humane methods, one could argue they are comparatively better off than many of their wild counterparts, living short lives under harsh conditions, and dying painful or violent deaths. And although ascertaining what a life worth having is remains philosophically challenging, I will make the case that it is more easily achieved if severe suffering is prevented, and refinement of husbandry, handling, procedures, and euthanasia are given top priority.

Presentation: Oral

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What can possibly go wrong?

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This talk and subsequent discussion of educational material will concentrate on how to convey to new researchers the problems that have been identified in the conducting and reporting of preclinical studies, and how to avoid them, and on the basic measures to make experiments more reliable, robust and reproducible. Claims of low predictive value of animal experiments based on their results not translating into the expected effects in clinical trials may well have missed that such experiments would not have been reproducible in animals under similar circumstances, let alone in another species (human patients). The problems noted also occur in "*in vitro*" studies, and low reproducibility will eventually undermine trust in the value of both non-animal and animal studies to inform on human outcomes. No amount of technological development can prevent the most common issues affecting the reliability of research, as these have an impact at a fundamental level. Also, as irreproducible research is prevalent in highly developed countries, and published every day in the most prestigious journals, this is not simply a matter of needing more money. New researchers need to understand the key measures for improving research, namely randomization, blinding, identifying the correct experimental unit, controlling for common biases, and using adequate sample sizes. Educating them on these issues should help address the inadmissible ethical cost of the waste of animals in low-quality, unreproducible preclinical studies, as well as the risk of distrust of "*in vitro*" testing because it is poorly conducted.

Presentation: Oral

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Replacing animal use in the acute toxicity "six-pack" for industrial chemicals and pesticides – US perspective

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The U.S. Environmental Protection Agency's Office of Chemical Safety and Pollution Prevention (OCSPP) is prioritizing efforts to reduce and replace vertebrate animal testing with established alternative methods that increase rigor in the agency's assessments and remain fully protective of human health and the environment. To provide transparency around the agency's efforts to replace *in vivo* tests, metrics are annually reported online; this presentation will provide the most recent data on these metrics. OCSPP is collaborating with federal partners and stakeholders to implement new approach methods (NAMs) shown to be more human relevant, scientifically valid, and reproducible than the traditional methods prescribed in the acute "six-pack". This presentation will highlight the implementation of several science policy and guidance documents. A recent analysis of the Global Harmonized System for Classification and Labeling (GHS) Mixtures Equation's utility for predicting acute oral toxicity hazard categories of pesticide formulations indicated an overall concordance of 55% for EPA classification when

compared to *in vivo* data. However, within-class concordance was highest in EPA Toxicity Category IV and GHS Category 5 at 87% and 88% respectively, thus demonstrating the Mixtures Equation may be able to identify substances with lower potency. A framework for prioritizing human-relevant *in vitro* data to predict irritation potential of new chemical substances and defined approaches for hazard classification of pesticides utilizing several OECD accepted *in vitro* test guidelines will be presented. Similarly, efforts to determine the applicability of multiple *in vitro* and *ex vivo* tests to identify skin irritation hazard are ongoing.

Presentation: Oral

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Brain organoids for developmental neurotoxicity testing and gene / environment interactions (GxE)

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The human brain represents the most complex organ of our body and thus modeling a development of functioning human brain-on-dish is an enormous challenge. Recent advances of microphysiological systems are a way to address brain development, homeostasis, functionality and diseases in more physiologically and human-relevant conditions. Already a simple brain spheroid represents higher complexity, increased cell-to-cell interactions, prolonged shelf-life than monolayer cultures, offering an opportunity to conduct longitudinal studies on chemicals affecting neural development. Brain organoids allow addressing the histoarchitecture and cellular interactions of the brain including synaptogenesis and myelination. Using CRISPR/Cas9 gene-editing technology in iPSC, we have developed multi-fluorescent brain organoids, which allowed to screen environmental chemicals and drugs in a complex brain model. To bring brain organoids closer to *in vivo*, we introduced iPSC-derived microglia, immune-cells of the brain, which allowed us to study not only the role of microglia in synaptogenesis but also neuroinflammation upon chemical exposure and viral infections. We recently developed a 3D multi-electrode array platform (organoid EEG). We use this EEGs and high-density multi-electrode arrays to develop assays for learning and memory. This should fill the gap in the functional endpoints in the *in vitro* developmental neurotoxicity testing battery. Finally, we used brain organoids with autism genetic background to address gene environmental interactions in autism and validated the findings against human data, providing a first example of *in vitro* GxE and a mechanistic validation concept.

Presentation: Oral

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Training and courses offered at Johns Hopkins Center for Alternatives to Animal Testing

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The Center for Alternatives to Animal Testing (CAAT) has been for 42 years the leading think tank for alternative methods as new approach methodologies (NAMs) in safety sciences in the US. Research at CAAT addresses mechanistic toxicology including a complete metabolomics workflow, microphysiological systems (MPS) of the brain and their implementations in the field of developmental neurotoxicology to replace animal testing. The CAAT laboratory provides a platform for young scientists from around the world to learn and apply NAMs and interact with researchers in the field. Beyond Johns Hopkins students, CAAT welcomes international fellows and supports internships. Besides hands-on training, CAAT promotes NAMs through teaching. At JHU, CAAT offers courses on Toxicology of 21st Century, Evidence-based Toxicology, Policy and Humane science for animals in research, and bioinformatic tools in toxicology. CAAT faculty offers a class on Introduction in Alternatives Methods at Georgetown University as a part of Environmental Metrology and Policy program. Importantly, since 2018, courses on Toxicology of 21st Century and Evidence-based Toxicology have been offered on COURSERA, an open online learning platform. Since then, these two classes have attracted almost 20,000 active learners. Translational activities of CAAT include workshops (transatlantic think tank for toxicology, t^4) and the secretariats for the Evidence-based Toxicology Collaboration, the Good Cell Culture Practice Collaboration, the Green Toxicology Collaboration and the Industry Refinement Working Group. Recently, CAAT was awarded a NIH/NCATS grant to establish a series of MPS world summits and international MPS society.

Presentation: Oral

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Methods2AOP: An international collaboration advancing AOP key event descriptions

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The AOP framework requires that key events (KEs) be quantifiable to substantiate linkages between stressors and effects. The existing AOP-Wiki section for method-related information ("how it is measured or detected") neither reflects the importance of linking KE with test method nor enables consistent description of methods across KEs. Methods2AOP is an international collaboration aiming to make linkages between test methods and KEs more explicit and visible. Collaborators have identified roughly 30 information fields across two levels to associate test method with KEs (level 1: critical) in a simple but FAIR (Findable, Accessible, Interoperable, Reusable) manner while also integrating sufficient assay details (level 2: informational) to annotate technical implementation and interpretation of results in AOP context. Fields drawn from established ontologies will be tabularized in the AOP-Wiki. The goal is for test method tables to formalize connections between AOP descriptions, external sources, and supporting evidence. Encouraging adoption among contributors is essential to collecting requisite details to increase the overall trustworthiness and utility of a methods-annotated AOP. This annotation framework is aligned with requirements from stakeholders including OECD EAGMST to advance the integration of experimental definitions into AOP frameworks and facilitate regulatory relevance of AOP knowledge. Facilitated by JRC, EURL ECVAM in collaboration with NIH NICEATM, US EPA, Environment and Climate Change Canada, and others.

The views expressed in this presentation are the authors own and do not necessarily reflect those of the US government; funded by NIEHS, NIH under Contract No. HHSN273201500010C.

Presentation: Oral

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Toward probabilistic risk assessment – The ONTOX project

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Safety sciences must cope with uncertainty of models and results as well as information gaps. Acknowledging this uncertainty necessitates embracing probabilities and accepting the remaining risk. Every toxicological tool delivers only probable results. Traditionally, this is taken into account by using uncertainty / assessment factors and worst-case / precautionary approaches and thresholds. Probabilistic methods and Bayesian approaches seek to characterize these uncertainties and promise to support better risk assessment and, thereby, improve risk management decisions. Actual assessments of uncertainty can be more realistic than worst-case scenarios and may allow less conservative safety margins. Most importantly, as soon as we agree on uncertainty, this defines room for improvement and allows a transition from traditional to new approach methods as an engineering exercise. The objective nature of these mathematical tools allows to assign each methodology its fair place in evidence integration, whether in the context of risk assessment, systematic reviews, or in the definition of an integrated testing strategy (ITS) / defined approach (DA) / integrated approach to testing and assessment (IATA). This presentation gives an overview of methods for probabilistic risk assessment and their application for exposure assessment, physiologically-based kinetic modelling, probability of hazard assessment (based on quantitative and read-across based structure-activity relationships, and mechanistic alerts from *in vitro* studies), individual susceptibility assessment, and evidence integration. Ongoing work in the context of the EU ONTOX project will be shared. In conclusion, probabilistic risk assessment will be key for constructing a new toxicology paradigm – probably!

Presentation: Oral

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Annotating high-throughput screening assays: Facilitating interpretation and data use

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Publicly available high-throughput screening (HTS) data have the potential to facilitate the development of computational approaches for chemical assessments and provide mechanistic insight on chemical effects and hazard. However, linking HTS data to toxicologically relevant mechanistic pathways or regulatory endpoints remains a challenge and requires detailed information about both assay technology and the assay's biological context. In this project, we annotated thousands of assay endpoints from the U.S. Environmental Protection Agency's ToxCast program, including results from the Tox21 HTS program, using existing controlled assay ontologies. Use of these ontologies facilitates stakeholder understanding, provides terminology that offers additional context, and informs upon the biological relevance of heterogeneous *in vitro* HTS assay readouts. Assay annotations are leveraged to complete a standardized data reporting template: OECD Harmonized Template (OHT) 201. OHT201 is an internationally recognized template used to report chemical test result summaries for intermediate effects. It captures assay technology information as well as mechanistic outcomes and interpretation obtained from *in vitro*, *ex vivo*, or *in silico* methods. We used a KNIME workflow applied via the IUCLID portal to populate the OHT201 form based on expert curation of existing annotations associated with fields in the form. These activities are expected to increase accessibility to annotated HTS data and provide context to facilitate the identification of data gaps, characterization of mechanistic plausibility, and further investigation into regulatory-relevant endpoints such as endocrine disruption, carcinogenicity, developmental toxicity, etc.

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Presentation: Oral

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Addressing the yearly $53B problem for pharma companies, using "Patient-on-a-chip" technology and innovative AI

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Drug discovery and development is a lengthy and expensive process with more than a 92% failure rate, costing pharma companies an astonishing $53B each year in failed animal testing, killing 110 million mice, and 50,000 beagles, every year. This topic is more relevant than ever- with the revolutionary legislation in the US removing the antiquated 83-year-old requirement to use animal studies as part of the process to obtain a license for a new drug. Quris is here to solve this problem. We've developed the first Bio-AI clinical-prediction platform that can better predict which drug candidate will be safe in the human body, and for whom, avoiding the massive cost and time of animal testing. Our BioAI platform

uniquely combines the power of cutting-edge AI together with patients-on-chip technology, to better predict drug safety. How does it work? A simple three-tiered process:

We generate millions of interactions between known drugs (safe and toxic drugs) and patients-on-chip (miniaturized interconnected human organs on a chip).

We Train the AI, based on the proprietary auto-labeled nano-sensor data extracted from these interactions.

This training then allows the platform to predict the safety and personalization of new drug candidates accurately.

This integration of state-of-the-art machine learning, with patient-on-a-chip, real-time nano-sensing, and stem-cell genomic diversity technologies- allows high indication of drug toxicity. We are at the tipping point of the modernization of drug discovery-Quris' platform could be of very significant value to pharma companies and the health of society at large.

Presentation: Oral

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Is it time for Tox-21c 2.0 thanks to AI?

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The 2007 NRC report on Toxicity Testing for the 21st Century – a vision and a strategy (Tox-21c) was a watershed moment for US toxicology changing a discussion from whether to change to when and how? With knowledge vastly increased since 2007, technologies such as microphysiological systems (MPS) and especially artificial intelligence (AI) have emerged, which were hardly covered in the report. Exposure-driven assessments were only covered in a parallel NRC report but the needs for integration into toxicology, for example, through exposomics, are increasingly evident. A key challenge is the integration of data and methods (evidence streams) in Test Strategies, Systematic Reviews, and Risk Assessments. Evidence-based Toxicology and Probabilistic Risk Assessments are emerging here. In order to embrace these developments and move Tox-21c toward implementation, the Basic Research Office of the Under Secretary of Defense for Research and Engineering, OUSD(R&E), hosted a Future Directions workshop Advancing the Next Scientific Revolution in Toxicology on April 28-29, 2022, at the Basic Research Innovation Collaboration Center (BRICC), in Arlington, VA. A vanguard of Tox-21c scientists and agency observers developed a report laying out how recent developments can be embraced and Tox-21c implemented in the next decades. AI plays a key role in all aspects of this vision.

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A call for a human exposome project

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Four decades of the Human Genome Project and its consequences have shown how the entrepreneurial state, through significant investment into science, can drive scientific progress and advance biomedicine. A certain fraction of diseases can now be explained as caused by genetics, and a more significant fraction as impacted by genetics. Besides another fraction caused by pathogens, the third and probably largest impactor is exposure, i.e., the many physicochemical and lifestyle factors. This presentation makes the case that it is time to start a Human Exposome Project, which systematically explores and catalogs the exposure side of human health and disease. The envisioned Human Exposome Project needs to be more than a scaled exposomics approach, aiming to assess the totality of relevant exposures through ~omics of human body fluids and forming exposure hypotheses. Exposomics is increasingly complemented by exposure science and biomonitoring to measure exposure, mechanistic understanding, human-relevant microphysiological systems, big data, and artificial intelligence (AI) to mine these data and integrate pieces of evidence. The potential impact of AI on a possible Human Exposome Project is so substantial that we should speak of exposome intelligence (EI) because this allows us to expand our limited current knowledge to the big unknown unknowns of threats to human health.

Presentation: Oral

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Engagement of scientists with the public and policymakers to promote alternative methods

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Scientists are usually good at teaching, sometimes even to lay audiences. But communicating with journalists, activists, or policymakers can be a different story – hesitancy to make mistakes as well as the temptation to disproportionally promote one's own case come into play. The multitude of social media and other web-based outlets has diversified and accelerated the communication of science. Real-time reactions, sharing of data, tools and results, increasing invitation of personal opinion, demand for transparency, political correctness, and loss of trust in experts are challenges to

researchers in general. The field of alternatives to animal testing is more political and important to lay audiences than many others, so its scientists must be especially aware of these challenges. Public engagement offers the opportunity to form a community and create wide support for non-animal research and its implementation. This requires scientists to step out of the ivory tower of higher education and engage with diverse interest groups by outreach activities, interviews, press releases, etc., by employing tailored communication.

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Good Cell Culture Practice (GCCP) 2.0 extending to microphysiological systems

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A number of cell culture technologies have become more broadly available with the turn of the century, which allow overcoming shortcomings of traditional culture. These include the use of stem-cell derived cells, cocultures of different cell types, scaffolds and extracellular matrices, perfusion, 3D culture, tissue architecture and organ functionality. The physiological relevance is further enhanced by the measurement of biomarkers (e.g., key events of pathways) and more wholesome assessment cell responses by high-content methods. These approaches are still rarely combined to create microphysiological systems and in fact we do not argue that all cell culture needs to be that sophisticated. However, the less defined the endpoint of interest and cellular response are, the better we should approximate organ- or tissue-like culture conditions to make physiological responses more probable. Beside these technologic advances, important progress in the quality assurance and reporting on cell cultures as well as the validation of cellular test systems bring the utility of cell cultures to a new level. Lessons learned from the development, validation and acceptance of alternative methods for the creation of a new approach for research and especially regulatory toxicology show that we need both conceptual steering and a quality assurance. Since 2022, Good Cell Culture Practice (GCCP) 2.0 is available, which expands the original GCCP guidance from 2005 to the new approaches.

Presentation: Oral

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The progress of alternative methods in Brazil: Activities of BraCVAM

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BraCVAM, the Brazilian Centre fof Validation of Alternative Methods, was created in 2013 and is based in the Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. BraCVAM is linked to the Vice-Presidency of Research and Biological Colletion of FIOCRUZ. Along this period of time BraCVAM has suggested the officialization of more than 30 alternative methods to the National Council for the Control of Animal Experimentation (CONCEA) that published 4 Normative Resolutions on this issue. Members of BraCVAM coordinate the first "Latu sensu" post-graduation course, entitled Specialization on Alternative Methods on Animal Use, in FIOCRUZ, with 3 classes formed. BraCVAM is also responsible for the academic discipline of Alternative Methods in the master's degree course on laboratory animal science (MPCAL), in FIOCRUZ. BraCVAM has also participate in organization of editions of the Latin American Congress of Alternative Methods (COLAMA), besides presenting many abstracts, posters, classes and lectures in a variety of events in Brazil and worldwide. BraCVAM established a partnership with Humane Society International (HSI) for disseminating the Monocyte Activation Test (MAT). The most recent action of BraCVAM was to suggest the Brazilian Pharmacopoeia the adoption of MAT as an official monograph for replacing rabbits in the pyrogenicity testing, which was accepted by the Pharmacopeia and BraCVAM was invited to participate in the working group that will include MAT as general monograph.

Presentation: Oral

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Unleashing the potential of bioprinted tumors: A step towards personalized cancer therapy

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One of the major challenges in cancer drug development is the lack of effective models during preclinical testing. The available models do not mimic the human *in vivo* microenvironment. Further, pre-clinical testing is heavily dependent on animals, and this inflicts both psychological and physiological stress on animals. A large number of animals are exposed to potential anti-cancer agents on a daily basis and eventually, only 5% of these compounds move towards human clinical trials. This issue poses a question on the relevance of using animals for modeling "human" cancer. To solve this issue, we propose the use of "Bioprinted Tumor Models" as an alternative to animal models. In this study, using bioprinting (3D printing) technology, we have created a unique tumor tissue that closely mimics the human tumor microenvironment. In order to achieve this, we have created a specialized bioink which to our knowledge remains the most human tumor-specific bioink. Most bioinks used for bioprinting are derived from rodents (e.g. Matrigel), thus contrasting the purpose of replacing or reducing the use of animals from an ethical standpoint. Our model is free of any animals derived material thus clearly aligning it with the "3Rs" principle. We have extensively characterized the tumors in terms of cell viability, tumor stiffness, cell morphology, and structure etc. Further, we have also tested standard chemotherapies, radiation therapy, and immunotherapy. Our vision is focused on creating and scaling the bioprinted tumor model as a means of testing anti-cancer therapies in both academia and the industrial sector.

Presentation: Oral

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The DNT IVB – A challenging road leading to change

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For developmental neurotoxicity (DNT) evaluation, *in vitro* methods allow a more efficient testing for hazard identification compared to traditional animal experiments. Recently, a DNT-*in vitro* battery (DNT IVB) has been compiled under international regulatory guidance, which is the sum of its single assays and represents a large variety of neurodevelopmental key events like human neural progenitor cell (hNPC) proliferation, their neural differentiation, migration and maturation. An OECD DNT IVB guidance document is being composed that should facilitate IVB interpretation.

To gain confidence in the DNT IVB, we pursued four strategies with the EU part of the DNT IVB. First, within the European H2020 project ONTOX we are generating physiological maps for brain development. These maps are then compared to the current DTN IVB. Secondly, we employed molecules altering signalling pathways known to be crucial in brain development/human neurodevelopmental diseases. Results from both efforts inform on IVB gaps. Third, we calculated battery performance based on chemical testing of a reference set of 46 positive and negative DNT compounds. Last, we strengthen the relevance of IVB hits by assembling transcriptome-supported DNT adverse outcome pathways (AOPs).

In summary, an international effort led to the first generation DNT IVB. Gap and strength analyses reveal areas where more scientific input is needed. The collective work of the scientists involved will increase confidence in the battery and help decrease uncertainty for its regulatory application.

Presentation: Oral

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ETPLAS for the benefit of free movement of researchers and harmonisation of quality standards across Europe

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The European Directive 2010/63/EU, in articles 23 and 24 requires competence of all personnel involved in the use of animals for scientific purposes. The Education and Training Platform for Laboratory Animal Science, ETPLAS, was set up to aid harmonisation and mutual acceptance of LAS training across the EU. Today, ETPLAS offers open access training materials for animal care staff, Competent Authorities, and training providers. ETPLAS' training toolbox will continue to increase in the coming years. Building on from the European Parliament funded Pilot-Project (2019-2021), the EU funded Action-Project continued under the ETPLAS umbrella in 2022. The Pilot-Project focussed on Core and function A (those carrying out procedures on animals) specific modules. The scope of the Action-Project is extended to include functions B (those designing procedures and projects) and C (those taking care of animals) specific modules. Four Working Groups are focussing on expanding the training assessment criteria; preparing a Question Database for assessment through an online examination service that will be made available to all LAS training providers; extending the Direct Observation of Procedural Skills (DOPS) database and developing a EU wide continuing professional development (CPD) framework for the field of Laboratory Animal Science (LAS) thereby facilitating a harmonised approach to the maintenance of competence, as required by the EU-Directive.

The way ETPLAS will continue to develop further quality standards for mutual acceptance of Education and Training in LAS across Europe will be expanded upon.

Presentation: Oral

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Multimodal welfare assessment in laboratory mice after surgery

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The best possible animal welfare during experimental procedures is fundamental for high data quality in biomedical research. It is the basis for the generalizability, robustness, and reproducibility of results, and its assessment requires an objective and exact approach. This study aimed to evaluate a multimodal welfare assessment after surgery in mice.

After intraperitoneal implantation of a telemetric device into female C57BL/6J mice, post-operative multimodal welfare assessment included clinical scoring of general appearance and body weight, the monitoring of telemetry-derived heart rate (HR), heart rate variability (HRV), temperature and activity and the assessment of the pain-specific parameter the Mouse Grimace Scale (MGS) and burrowing behavior.

After surgery, general appearance remained unaffected, but body weights were significantly reduced for up to one week. In addition, telemetry-derived HR was increased, and HRV and activity were decreased for up to 2 weeks after surgery. Body core temperature was also not affected. In contrast, the pain-specific parameter MGS was increased only hours after implantation, and burrowing behavior was decreased for less than a week.

These findings revealed that the different modalities show different periods of impaired welfare, with an indication of short-lasting pain but longer-lasting impairment due to the altered heart function and reduced activity. Moreover, it can be suggested that the different modalities indicate different facets of impairment, such as pain or discomfort only. However, the results support the conclusion that only the composition of multiple parameters reveals a holistic picture of the overall impairment after surgery in laboratory mice.

Presentation: Oral

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Targeting respiratory viruses: A novel alveolus-on-chip infection model for pre-clinical applications

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Respiratory viruses pose a constant threat to public health as highlighted by the COVID-19 pandemic. In the distal lung, SARS-CoV-2 infection results in air-blood barrier breakdown and massive inflammation in severe cases. Due to the difficulty to model the alveoli, animal models are widely used. However, they do not capture all aspects of severe COVID-19, which complicates translation of pre-clinical findings into clinical practice. Human-derived microphysiological systems enable to study the multifactorial pathophysiology of COVID-19 in a human-relevant context and potentially fuel drug development.

Therefore, we aimed to develop AlvireX, a drug screening platform for respiratory viruses. It combines medium-throughput screening on alveolar microtissues and validation on a lung-on-chip model.

We used an alveolar epithelial cell line (AXiAEC) derived from primary human alveolar epithelial cells and optimized for on-chip applications. AXiAEC were infected with SARS-CoV-2 or influenza A virus (IAV) and monitored for virus production and barrier function.

In air-liquid interface (ALI), AXiAEC upregulated host factors for SARS-CoV-2 cell entry. Consequently, SARS-CoV-2 infected AXiAEC and induced barrier breakdown. Physiological stretch promoted SARS-CoV-2 production. Finally, we demonstrated productive IAV infection in AXiAEC. IAV replicated more rapidly than SARS-CoV-2, which recapitulates their differential incubation time in patients (SARS-CoV-2: ~5.5 days; IAV: ~2 days).

Our results highlight the importance of replicating the complex physiological microenvironment (ALI, stretch) to model viral infection and immune response in the alveoli. We believe that the AlvireX platform will be broadly applicable to current and emerging respiratory viruses and will help accelerate drug testing in the pre-clinical phase.

Presentation: Oral

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Evaluating new approach methodologies for use in next generation risk assessment

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There is an increasing recognition [1] that new frameworks are required for establishing scientific confidence in the use of New Approach Methodologies (NAMs) for regulatory purposes. This is especially needed in the context of Next Generation Risk Assessment (NGRA), which is an exposure led and hypothesis driven approach for conducting safety assessments using non-animal NAMs. A key requirement for the acceptance of NAM-based toolboxes and workflows for use in safety assessments is that they are fit-for-purpose, which requires that they are evaluated in the context of their use (i.e., risk assessment). To address this, we recently proposed a NAM-based toolbox and workflow for conducting systemic safety assessments, together with an approach for evaluating how protective it is [2]. The approach is based on the principle of benchmarking safety decisions made using the NAM toolbox against historical safety decisions. The toolbox includes physiologically-based kinetic (PBK) models and a broad range of different *in vitro* assays, from which points of departure (PODs) are estimated. The feasibility of the evaluation strategy was initially tested using 24 benchmark exposure scenarios from 10 different chemicals [2] and has since been expanded to include 69 additional benchmark exposure scenarios from 38 additional chemicals. In this talk, I will present the results of the extended evaluation, with a particular focus on how our evaluation approach aligns with current proposed changes to wider validation frameworks.

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Presentation: Oral

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Cross-taxa predictive ecotoxicology: Data, splitting, performance

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Much effort in predictive toxicology has been focused on mammals. In contrast, similar efforts are surprisingly limited in ecotoxicology despite the fact that it still heavily relies on animal testing for, e.g., chemical hazard assessment, which not only requires animal sacrifices but is also very resource-intensive. Therefore, our research is aimed at exploring the potential and limitations of applying machine learning (ML) to ecotoxicology. Previous studies are usually limited in taxonomic and chemical scope (datasets on single or few species and few chemicals). Therefore, we compiled an extensive dataset aimed at acute mortality and related endpoints in three distinct taxonomic groups of high relevance in ecotoxicology (fish, crustaceans, and algae), covering ~2400 chemicals and over 1200 species. The core dataset of ecotoxicity experiments was supplemented by phylogenetic and species-specific features, as well as chemical properties, and molecular representations. We provide the dataset including an in-depth characterization of all features and the reasoning behind the feature curation to serve as a benchmark. To complete the benchmark dataset, we propose a number of prediction challenges that could help answer pressing questions from our field and give options for different splitting approaches to prevent data leakage. We compare model performances of several regression-based ML methods on the dataset, with special consideration towards exploring the influence of different train-test-splits, feature importance, and molecular representations.

Presentation: Oral

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The role of qAOPs in exposure-led NGRA: Benefits and limitations

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Next generation risk assessment (NGRA) is 1) exposure-led, 2) hypothesis driven, 3) uses a tiered and iterative approach to make safety decisions and 4) is designed to prevent harm. NGRA provides a workable framework to integrate exposure information and points of departure from non-animal *in vitro* cell assays. When no bioactivity is expected at human-relevant concentrations, a safety decision can be made on the basis that no bioactivity indicates there can be no adversity. This type of safety assessment is protective of human health without predicting hazards that may occur at higher exposures. However, where bioactivity cannot be ruled out at human-relevant concentrations, higher-tier tools are needed to refine the risk assessment. In this talk, I will discuss how quantitative AOPs can in principle be useful for risk assessment decision making, when specific mechanisms of concern have been identified. This is particularly critical when distinguishing between adaptive and adverse effects associated with key event in an AOP. In the talk, the benefits and limitations of qAOPs and how they may be useful within the context of NGRA will be illustrated by drawing on several examples, including inhalation (inflammation and fibrosis) and systemic toxicity (Nrf2 activation and oxidative stress). The qAOP modelling approaches used in these examples will vary from systems biology (i.e., bottom up, mechanistic) to Bayesian statistical models. An industry perspective on the short- and long-term future challenges of how these different approaches could be selectively deployed within a tiered risk assessment framework will also be discussed.

Presentation: Oral

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Leveraging data science for better severity assessment in laboratory animal science

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Data Science has revolutionized laboratory animal science, particularly in severity assessment, which is critical for ensuring the ethical and humane treatment of animals in research. With the increasing use of animals in biomedical research, new methods and techniques are being developed to assess the severity of procedures and minimize pain and distress. In this regard, supervised and unsupervised Machine Learning methods and algorithmic design have emerged as new and innovative tools to assist researchers and routine workers in decision-making.

To this end, the DFG-funded research group "FOR2591 Severity Assessment" has developed several tools and algorithms to analyze clinical, physiological, and behavioral data. These tools included methods of comparative severity assessment (RELSA), humane endpoint estimation (endpointR), severity classification (lauRa), and an application for finding the optimal representation of multiple severity-related variables in animal models (CMS). Ultimately, combining quantitative solutions with laboratory animal science led to a higher quality severity assessment, resulting in new ways of ranking the severity of procedures and finding better humane endpoints.

The goal was to use Data Science to improve animal welfare and promote ethical and responsible research. However, it remains crucial to validate these methods and establish clear guidelines to ensure robustness and generalizability. With the rise of AI technologies, laboratory animal science is undergoing a transformative period. Researchers and routine workers must be prepared to adapt to these new tools and establish trust in their use. By doing so, we can work towards a future in which animal research is conducted more ethically and humanely.

Presentation: Oral

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The RELSA score: An evidence-based tool for better severity assessment in animal research

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The severity assessment of laboratory animals is crucial for reliable and ethical animal research. However, determining the most objective way to assess animal well-being remains challenging. Traditional manual scoring of animals is subjective and can be influenced by factors such as training, motivation, and external conditions. As a result, there is a growing need for more reliable and objective approaches to severity assessment.

To address this issue, we have developed the Relative Severity Assessment (RELSA) score based on a comprehensive pipeline using physiological and behavioral data. These data were mapped to a single value representing animal well-being on a relative scale, making the multidimensional nature of severity transparent and allowing for more objective monitoring. We could classify animals and animal models into distinct categories, allowing us to rank and validate their severity, e.g., sepsis > surgery > restraint stress > colitis. This approach was extended to four gastrointestinal studies for which the order BDL > Pancreatitis ≈ PDA > CCl4 was retained, when the RELSA reference data came from an independent laboratory. Therefore, we expanded the robust RELSA workflow to other animal models such as genetic, stress-based, and pharmacological mouse models of depression.

RELSA enables stakeholders to make retrospective, quantitative severity comparisons using the multidimensional fingerprint of the outcome measures and to perform prospective severity estimations for newly planned studies, providing evidence-based monitoring of animal well-being that can aid researchers in making better-informed decisions based on a more holistic representation of the animal on a quantitative scale.

Presentation: Oral

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Application of the GARDskin medical device assay for regulatory approval of medical devices according to MDR

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The assessment of skin sensitizers in medical device extracts is conventionally performed *in vivo*, primarily using the Guinea Pig Maximization Test and the Buehler Occluded Patch Test. However, the medical device toxicology is currently transitioning to a process which is increasingly focused on the use of *in vitro* methods for evaluation of the biological safety of medical devices. The recent inclusion of *in vitro* methods for the endpoints skin irritation and skin sensitization in the ISO 10993 standard now makes it possible to perform this testing *in vitro*. One of the *in vitro* methods for assessment of skin sensitization described in ISO 10993-10 is the GARDskin assay. The GARDskin assay is the first OECD TG 442 method that has been adapted to work with oil, the non-polar extraction vehicle often used in *in vivo* studies for testing medical devices.

Here we show an example of how *in vitro* results, including results from the GARDskin Medical Device assay, were submitted to obtain CE-marking according to the European Medical Device Regulation 2017/745 (MDR) for Tinearity® G1, an innovative tinnitus treatment medical device. The classification of Tinearity® G1 as a non-sensitizer in both polar and non-polar extracts in the GARDskin Medical Device assay was used with *in vitro* results for cytotoxicity and skin irritation as weight of evidence together with toxicological evaluation of chemical data in the risk assessment and biological evaluation of the medical device.

Presentation: Oral

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Practical application of new approach methods in developmental and reproductive toxicity (DART) testing

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Encouraged by the successful application of New Approach Methodologies (NAMs) in an exposure-driven Next Generation Risk Assessment (NGRA) approach to systemic toxicity (Middleton et al., 2022), an integrated framework with additional NAMs covering endpoints for developmental and reproductive toxicity (DART) was developed. This approach comprised DART-specific *in silico* predictions, *in vitro* physiologically based kinetic (PBK) modelling, a cell stress panel, high-throughput transcriptomics, *in vitro* pharmacological profiling as well as ReproTracker® and devTOX quickPredict™ to address developmental toxicity. To determine if this approach is sufficiently protective for consumer safety assessments, first its biological coverage was evaluated. The comparison between reported cellular processes, signaling pathways and genes involved in known key stages in human reproduction and embryo-fetal development to the read-outs from our NAM toolbox showed > 80% coverage based on gene numbers (Rajagopal et al., 2022). Approximately 40 benchmark substances were tested in all NAMs, and points of departure (PoDs) were compared to exposure estimates (plasma Cmax) obtained from published clinical studies (where available, or using PBK modelling) to calculate bioactivity-exposure ratios (BERs). To evaluate the approach, these BERs together with *in silico* predictions were compared to literature reports of the DART risk of each compound. These evaluations will be presented together with areas identified where integration of additional NAMS could be beneficial to enable human-relevant safety decisions. These areas include addition of placenta transfer models to the PBK modelling strategy, Calux® Assays to refine information on receptor binding, and use of other advanced cell models.

Presentation: Oral

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EU roadmap towards full replacement of animal-testing for industrial chemicals

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The European Commission supported by the European Chemicals Agency (ECHA) is working towards a roadmap for replacing animal testing for chemical safety in Europe. This follows recent announcements of roadmaps and plans to support the implementation of NAMs instead of animal testing by others (e.g., US EPA, EFSA) and the request for such a roadmap by citizens to the European Parliament and submitted via a parliamentary resolution in 2021.

The European legislative approach to industrial chemicals is unique in being a horizontal generic system for all chemicals which puts the burden of proof on industry. This creates a higher demand for universally applicable NAM solutions. The in-going assumption in the roadmap development is that the current horizontal generic system would be maintained, i.e., the identification of hazards through information requirements in the REACH Regulation and classification of substances based on adverse effects, by applying specific criteria agreed at EU and international level (GHS). The roadmap will spell out the critical needs necessary to transit to an animal free system like, e.g., the ability to derive toxicological reference values from molecular data as opposed to from adverse effects observed *in vivo*. The roadmap will also highlight areas where methodological developments of NAMs are still needed to achieve the desired end-goal and it will point out where elements of the current horizontal system may need to be adjusted in order to allow the use of NAM-data (e.g., hazard classes).

Presentation: Oral

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A fourth R – Reframing research to redefine refinement

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Several requirements and recommendations exist surrounding the use of animals in research and testing, including guidance documents, regulatory requirements, facility accreditation, formal audit and inspection, and discussion between experts and the public. Additionally, the "Culture of Care" is embedded in European Directive 2010/63/EU, which seeks to protect animals used for scientific purposes. These, collectively, should contribute to adoption of minimal standards of care for animals in laboratories. However, consideration of animals remains distinct from wider evaluation of the research question and the "suitability" of animals to address this. For example, guidance documents for drug development may permit, or even encourage, the use of "alternatives", but the documents are often drafted from a position of animals as the gold standard and thus refer to the use of animals throughout.

Here we consider what is needed to further encourage application of non-animal tools, focusing on drug development. We present a tool that combines animal welfare considerations with deeper evaluation of non-animal approaches. This "framework" is a straightforward questionnaire that could be answered by, for example, animal license holders or principal investigators. It combines elements of existing requirements with questions on the use of non-animal, new approach methodologies (NAMs), and includes suggestions for improvements necessary to allow establishments to reach a maximum score. Completion of four key performance indicators returns a numerical score that could be used to rank facilities. This ranking of facilities may be useful for investors, and for pharmaceutical companies that want to outsource research whilst maintaining minimal animal use.

Presentation: Oral

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Misinformation, disinformation, hyperbole, and exaggeration: Communicating the truth in the fake news era

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There are approximately 2.5 million new scientific papers published every year, and reports of researchers feeling overwhelmed by the sheer volume of research to keep up with. Couple this with misleading news media headlines like "Cancer cured" and "Dementia beaten" and it is hard to know what to believe!

Taking dementia as an example, this presentation will look at how to detect "fake news" and, consider how, as scientists, we can improve our reporting to prevent misinterpretation and overestimation of scientific studies. We will focus on assessing the costs of this distortion of science, taking account of the financial implications, impact on society and the waste of animal lives; all of which combine to ultimately reduce public trust in science. We offer some thoughts on how to address these issues to improve accuracy and restore faith.

Presentation: Oral

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An integral approach to reduce the use of fetal calf serum

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To stimulate the development and uptake of non-animal or new approach methods (NAMs), the 3Rs Centre Utrecht (3RCU) aims to maximally facilitate researchers to do so via integral approaches.

When embracing *in vitro* methodology, the use of fetal calf serum (FCS) confronts researchers with ethical and scientific obstacles. We are therefore aiming to replace/reduce the use of FCS. Our strategy encompasses:

- hosting a searchable, public database of available products, strategies and information to replace FCS
- organizing an active working group that meets regularly (once every 6-8 weeks). Members freely exchange information and have access to a shared environment containing relevant information
- match-making by hosting a database of interested and motivated students to be employed as interns and matching with interested researchers
- central coordination of storage and supply of replacement products (which are made available for free). This also ensures that everybody is working with the same reagents
- organization of lab space – if this is a limiting factor
- using social media for targeted campaigns to create attention, awareness and adherence. We have e.g. recently run a campaign specifically targeted at researchers that provided weekly scientific arguments against using FCS.

We will present a detailed plan of this strategy as well as the first results. Overall, our philosophy is that "creating data in addition to ideas" is the best way to stimulate researchers themselves to take up NAMs.

Presentation: Oral

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TXG-MAPr tools: Gene co-expression network analysis of toxicogenomic data to provide quantitative mode-of-action assessment and prediction of drug-induced toxicity

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Next-generation risk assessment (NGRA) of chemicals revolves around the use of mechanistic information without animal experimentation. Toxicogenomics is a powerful method to elucidate underlying mechanisms of chemical-induced toxicities, especially when considering the pathway or co-regulated gene network level. We developed the interactive TXG-MAPr tool, by applying weighted gene co-expression network analysis (WGCNA) on *in-vitro* liver and kidney datasets of primary human hepatocytes, HepG2 and RPTEC/TERT1 cells, as well as *in-vivo* rat liver and kidney data. The TXG-MAPr allows visualization of dose- and time-response data, compound correlation and functional annotation of gene networks (gene-ontology, pathway, transcription factor enrichment) to derive mechanistic information on mode-of-action. Perturbations of WGCNA gene networks were quantitatively assessed by module eigengene scores (EGs). Module EGs were associated with pathology phenotypes, providing prognostic information for drug safety assessment. In addition, new module EGs can be obtained by uploading transcriptomics data into the TXG-MAPr tool, which can be applied to investigate novel mechanisms of toxicities by chemical insults. Benchmark concentrations can be derived from co-expression modules and can be used as point of departure (PoD) of co-expression networks or associated pathways. Finally, module preservation between test systems could identify networks that are preserved *in vitro* and are associated to an *in-vivo* pathology. These modules could be mapped to key events in an adverse outcome pathway, enabling hazard identification for NGRA purposes. Ergo, TXG-MAPr represents a powerful tool for NGRA, providing mechanistic understanding of potential adverse chemical reactions, and to determine the PoD of key events associated with cellular adversities.

Presentation: Oral

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Modeling the stages of cervical cancer pathogenesis: Establishment of a healthy cervix-, a pre-cancerous CIN- and an immunocompetent carcinoma-on-chip

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Infections of the female cervix (CX) with the sexually transmitted human papilloma virus (HPV) can lead to a pre-cancerous cervical intraepithelial neoplasia (CIN) and progress to a cervical cancer (CC), the 4th most common cancer within women. Hence, human-based, immunocompetent physiological *in vitro* models of cervical carcinoma pathogenesis are urgently required for basic research and for the development of (immuno)therapeutic options.

A tailored microfluidic platform is fabricated by combining layers of laser-cut and hot-embossed thermoplastics and thermoplastic elastomers. Keratinocytes, fibroblasts, and neutrophils are isolated from human cervical tissue and whole blood of healthy donors. Primary cells as well as the cervical cancer cell line SiHa are combined in dynamic co- or triple-cultures in a hydrogel or scaffold. Neutrophils are perfused through the channel. The tissues are characterized via on-chip immunofluorescent staining, effluent analysis, and off-chip histology.

The developed MPS incorporates two independent systems with four replicate tissue chambers each. The 3D stromal layer with primary fibroblasts supports the multi-layered epithelium of primary keratinocytes and SiHa for the healthy CX- and the CIN-on-chip respectively. In the CC-on-Chip, SiHa spheroids emulate cancerous nests that respond to co-culture with fibroblasts and to compound treatment. Neutrophils migrate into the cancerous tissue.

We established a novel design and fabrication concept for a hybrid-material MPS that allows the generation of physiological human micro-tissues of different stages in the development of cervical cancer. Further integration of immune components in these platforms will boost research in immune-oncology therapy [1].

Reference

[1] Kromidas, Maulana et al. (2021). *Adv Drug Deliv Rev*.

Presentation: Oral

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Prediction of developmental neurotoxicity using a read across approach

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In silico methods have been widely used to predict and evaluate the toxicity of chemicals. However, there are not enough publicly available databases of animal studies for chemicals with complex toxicity mechanisms, such as neurotoxicity and developmental neurotoxicity. We have obtained *in vivo* developmental neurotoxicity datasets from literature information (such as *Neurotoxicol Teratol 52*: 25-35, 2015), and have been developing a new read-across method using molecular descriptors that represent physicochemical characteristics of chemical substances. We found that setting a threshold for the distance between substances used to define the neighbor substances and setting the number of neighbor substances to an appropriate value can increase the accuracy of developmental neurotoxicity evaluation using the read-across method with molecular descriptors. We found that there are chemical categories, which are correlated well with *in vivo* datasets. Other categories showed some discrepancies between *in silico* and *in vivo*. Because the discrepancies between *in silico* and *in vivo* may come from incomplete data of *in vivo* neurotoxicity, we have performed *in vitro* studies using iPSC/iPSC neurons to bridge the gap. Thus, the integrated approach using both *in silico* and *in vitro* would improve the prediction of developmental neurotoxicity *in vivo*.

Presentation: Oral

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Strategies to build a modular, cell culture-based approach toward the replacement of fish in environmental risk assessment

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Fish is the most widely used vertebrate in environmental risk assessment. Yet, current testing paradigms cannot live up to the demands of chemical safety assessment and the societal desire to reduce or replace animal testing. It is, therefore, our vision to build an alternative fish: a modular assemblage of fish cell line-based assays, alone or combined with computational models. Pursuing this vision, we have demonstrated that it is possible to use a rainbow trout (Oncorhynchus mykiss) cell line to predict the impact of chemicals on short-term survival and growth. Short-term survival is predicted using the RTgill-W1 cell line assay, assuming the gill to be the most common target site for acute fish toxicity. This assay has reached global acceptance as ISO standard 21115 and OECD test guideline 249; it hence comprises the first completed module of our envisioned alternative fish. Prediction of reduced fish growth, likewise, uses RTgill-W1 but measures cell proliferation as a proxy for fish cell number and consequently fish weight. An intestinal and a liver cell line are added as relevant sites for the derivation of chemical bioconcentration factors. We currently expand these concepts to modules for neurotoxicity (using a rainbow trout brain cell line) and reproductive toxicity (liver and gonad cell lines), and to subcellular markers with a documented link to adverse outcomes in fish. Our growing platform, therefore, should allow for the deployment of single or multiple modules of the alternative fish to reduce or, ideally, replace animal use in environmental risk assessment.

Presentation: Oral

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Novel assay to monitor phosphorylation-based signaling in cultured fish cells

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It is well known that proteins involved in cellular signaling cascades are often regulated by their (de)phosphorylation at specific sites, and many of the initiator kinases can respond to diverse stress signals, including those resulting from chemical exposure. However, mechanistic exploration of chemical effects on protein phosphorylation has received little attention in predictive (eco)toxicology so far, possibly due to technical limitations. Indeed, traditional antibody-based approaches to study protein phosphorylation can become prohibitively expensive when used in high-throughput assays and for multiple protein targets. Furthermore, suitable antibodies for proteins of interest are often lacking in non-mammalian species. To address this challenge, we set out to develop a targeted (phospho)proteomics method that would allow quantifying phosphorylation and abundance levels of multiple protein targets simultaneously within one mass spectrometry-based assay. We worked with the zebrafish (*Danio rerio*) PAC2 cell line model and focused on the mTOR kinase pathway, which is known to regulate growth and hypothesized to mediate chemical effects on growth. Currently, our method allows monitoring 19 protein targets along the mTOR pathway that provide insights into cell growth, proliferation, and energy regulation. We used it to monitor the mTOR pathway dynamics during (i) cell culture growth cycle and (ii) chemical exposures, in correlation with other cellular responses such as changes in ATP levels. Our work demonstrates the applicability of the mass spectrometry-based targeted (phospho)proteomics to study chemical disruption of phosphorylation-based signaling in different organisms, which could allow identifying novel mechanistic targets and exploring their suitability as predictive markers of chemical toxicity.

Presentation: Oral

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Team science: Support structures for robust evidence generation and meta-research

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Translation of biomedical discoveries to patient benefit is challenging with high attrition rates. To tackle this challenge, improving the robustness of preclinical evidence while considering clinically relevant endpoints and market opportunities is crucial.

However, preclinical scientists frequently face professional and structural barriers in adopting good practices. To address this, we established a research and support structure called Responsible PrecliniX as an integrative approach on the institutional level to assess and improve preclinical evidence toward increased reproducibility and predictive value of projects. As a central intervention instrument, we established a transparent, systematic consultation process with a metric to assess the robustness of a project at its core. It addresses validity in three domains: internal, external, and translational validity, alongside reliability and complements field-specific knowledge with statistics, experimental design, and meta-research expertise. Internal validity queries strategies to reduce bias, while external validity is concerned with strategies to establish the generalizability of results. Particularly, extending experimental designs to e.g., both sexes, different strains, or disease models. This may include the possibility of replications and non-animal alternatives. For translational validity, we assess how closely results reflect the clinical situation. Biostatistical advice is provided to ensure adequate reliability of generated evidence.

The process generates actionable items to improve the chances of (academic) projects reaching the clinic and being taken forward toward product development.

Uniquely, the metric and accompanying projects throughout the process will allow us to define key performance indicators to generate empirical evidence about the benefits as well as pitfalls of the translational process.

Presentation: Oral

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"Initiative Transparente Tierversuche" – The German initiative to promote transparent communication on animal research

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Trust in science is a valuable asset that can contribute significantly to social cohesion as well as to rational, democratic decision-making. Since trust is not a given good, but a joint effort based on openness, truthfulness and dialogue, it gives rise to a responsibility that radiates to science as a whole. In a controversial subject area such as animal experimental research, transparent information about scientific work and an open exchange of views on the choice of research methods that is open to dialogue are particularly important, as are ethical responsibilities. In this societal dialogue, the scientific community is called upon to continuously fulfill its central role and special responsibility. To promote open dialogue and transparency in animal research in Germany, the Permanent Senate Commission on Animal Protection and Experimentation of the German Research Foundation and the information initiative "*Tierversuche Verstehen*" of the Alliance of Scientific Organizations founded the "*Initiative Transparente Tierversuche*" ("Initiative on transparent animal research"). Comparably to other similar activities, the initiative formulates 4 goals which aim on fostering a culture of open dialogue and proactive information sharing. Universities, research institutions and industries are invited to join and support the initiative and implement their goals. The initiative was founded in summer 2021 and over 90 institutions have joined until early 2023. The initiative offers an annual workshop, a symposium, direct consultations and other resources to supporters of the initiative to help improve their own communication, outreach and transparency efforts.

Presentation: Oral

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The need of harmonization of alternative methods and animal use in Latin America

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Nowadays only Brazil, Uruguay and Mexico have laws regulating the use of animals in research and education. In the case of Brazil, the law 11,794/2008 also stimulates the application of the 3Rs in order to avoid any abusive experiment. It also obliges all institutions that use animals need to have an Ethical Committee for analyzing projects prior to begin experimentations. Several countries in Latin America have Ethical Committees but it is a voluntary initiative without law protection. Many other countries have law for animal welfare but that not necessary improve the use of 3Rs. This situation is very fragile in terms of disseminating alternative methods throughout the continent. It is necessary that the Governments of LATAM countries to aware of the need to take care of animals and, in priority, establishment of the 3Rs. For this harmonization the role of the Brazilian Council for Animal Experimentation Control (CONCEA), the Brazilian Center for Validation of Alternative Methods (BraCVAM), the Federation of South American Societies for Laboratory Animal Science (FESSACAL) and the Federation of North American Hispanic, Central America and Caribbean Societies and Associations for Laboratory Animal Science (FeSAHANCCCAL) is very important, specially by promoting meetings and congresses. In addition to acting politically in each country in order to adopt and publish laws that regulate animal use and improvement of the 3Rs application in research and education in Latin America countries.

Presentation: Oral

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Communicating with the general public about the science of animal research

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Several popular science books have been written in recent years about "sloppy science". Richard Harris, for example, wrote about research integrity and the "reproducibility crisis" in Rigor Mortis (2017), while Ben Goldacre's Bad Science (2008) and I Think You'll Find It's a Bit More Complicated Than That (2015) exposed problems with the science of medical research. All sold well.

Pandora Pound decided to try to write a similar book, aimed at the general public, about the science of animal research and the new approaches available to replace it. Wanting a wide readership, she found a literary agent to represent the book to mainstream publishers. The agent sent the proposal to editors at 30 large publishing houses, emphasising that the book was about science, innovative replacement technologies and contained no details of animal suffering.

The responses from publishers throw light on the way this topic is perceived by the print media. Although positive about the writing, none wanted to take it on. Most replied that the topic was too "niche" and that the book would not find a large enough market. Others felt the subject matter was a "tough sell", with the details "too difficult to stomach". A few suggested the topic would be better suited to a long-form article, or articles. Some editors' personal views about the value of animal research generated negative responses. Their feedback highlights the challenges of communicating with the general public – and the media – about the science and replacement of animal research.

Presentation: Oral

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Development of a cartridge bioreactor for parallelized cultivation and stimulation of complex tissue models

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The increased incidence of bone fractures represents a challenge for healthcare stakeholders. In the absence of sufficiently suitable *in vitro* models, animal models are still frequently used to analyze processes, interventions, and the success of fracture healing, especially in the preclinical phase. To address this gap, we have developed a 3D *in vitro* fracture healing (FH) model that simulates the initial phase of fracture healing by co-culturing fracture hematoma models with scaffold-free bone models. Although it operates under non-perfused and unloaded conditions, we have already observed a clear convergence between data obtained from *in vitro* and *ex/in vivo* specimens.

Knowing that loading and perfusion are essential factors for optimal bone regeneration, we here aim to develop a multimodal bioreactor capable of applying these parameters and allowing us to grow 3D FH models *in vitro* under controlled and constantly monitored conditions.

First, we specified mechanical loading conditions corresponding to the *in vivo* situation during bone regeneration. Next, we tested biomaterials and strategies for drug treatment under the specified loading and environmental parameters. Based on these data, we designed a 4-well insert-based Boyden chamber to incubate four FH models in parallel. We included a click-on pressure system to implement mechanical loading using a pneumatic valve. Using this system, we could prolong cell survival and accelerate the calcification process in our FH models.

The multimodal bioreactor proposed here will allow us to apply loading and perfusion as essential parameters for optimal bone regeneration to analyze processes, interventions, and the success of fracture healing.

Presentation: Oral

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Systems-biology modelling of steatosis and uncertainty quantification towards NGRA

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Within the EU ONTOX project, we are developing quantitative adverse outcome pathway (qAOP) models to advance human-health risk assessment. Our systems modelling qAOP framework aims to integrate data at various biological organizations, ranging from molecular/cellular to tissue/organ. For one case study on steatosis, we build quantitative models incorporating detailed biological networks underpinning lipid metabolism. Our systems modelling approaches can quantify both apical key events and generic cellular key events like cellular stress activation. To evaluate our model performance, we utilize public domain data e.g. from Tox21 and BioStudies. Furthermore, we develop our qAOP integration pipeline with additional data like *in-vitro* data curated from ongoing efforts within the ASPIS qAOP working group. Towards the Next Generation Risk Assessment (NGRA), our qAOP also integrates toxicokinetic (TK) modeling to offer an open-source tool (implemented in Open Systems Pharmacology Suite; www.open-systems-pharmacology.org) to predict response-response and exposure-effect relationship. To better assess the confidence in model prediction, we are developing advanced computational approaches to quantify uncertainty in qAOPs models. For the steatosis case study, we exemplify the uncertainty quantification results together with potential pitfalls from frequentist and Bayesian statistical approaches by integrating data synthesized from qAOP models. Perspective usage of the uncertainty quantification approaches with integrated TK-qAOP models will be discussed with real *in-vitro* data for steatosis, but also with a broader scope for other apical toxicological endpoints.

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Presentation: Oral

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In vitro screening of three UV stabilizers and a UV filter: Cytotoxicity, CYP1A activity, and mRNA expression in an immortalized embryonic double-crested cormorant cell line

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Ultraviolet (UV) stabilizers and UV filters are added to industrial and personal care products to absorb UV rays and prevent photo oxidation. UV stabilizers and filters have been detected in a range of environmental matrices, including high trophic level bird species (e.g. double-crested cormorant). Little is known about the toxicological properties of these compounds, especially in birds. Previously, we established and characterized an immortalized embryonic double-crested cormorant hepatic cell line, DCH22, for chemical screening. In the present study, DCH22 cells, cultured as 3-dimensional spheroids, were exposed to three UV stabilizers (UV-328, UV-329, UV-9) and a UV filter (BP-3) at nominal concentrations of 0.1 to 1000 µM to determine cell viability, CYP1A activity and changes in mRNA expression. Cell viability and CYP1A activity were measured using ATP concentration and ethoxyresorufin-O-deethylase (EROD) activity, respectively. A double-crested cormorant EcoToxChip (PCR array), comprising 384 genes, will be used to determine changes in mRNA expression. The UV stabilizers and UV filter elicited similar effects on cell viability with LC50 values > 100 µM. UV-9 and BP-3 exposure resulted in a concentration-dependent increase of CYP1A activity in DCH22 spheroids, while UV-328 and UV-329 did not. Concentration-dependent (0.1 to 100 µM) changes in mRNA expression will be evaluated for the compounds and the dose-response modelling program PROAST (RIVM) will be used to determine benchmark doses. Given the increased use and prevalence of these compounds, these findings will generate much needed toxicity data using an *in vitro* wildlife avian model.

Presentation: Oral

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Simulating mechanical forces during joint movement on cartilage in an animal-free bioreactor set-up

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Pathomechanisms of degenerative joint diseases such as osteoarthritis (OA) ultimately result in cartilage breakdown. The exact underlying mechanisms of both cause and progression still remain unclear. Besides the impact of metabolic components and architecture, mechanical forces are well-known as important modulators of joint health, while aberrant forces are primary etiological factors leading to cartilage degeneration.

Here, we aimed to (i) develop a long-lasting human *in vitro* 3D cartilage model using alternated perfused cultivation, (ii) apply (patho-)physiological mechanical forces – fluidic shear stress (FSS) – and (iii) simulate TNF-α-mediated cartilage degradation.

Bone marrow-derived mesenchymal stromal cells (MSCs) were used to develop the 3D cartilage model incubated in a bioreactor with a perfusion cycle that facilitates pathological (< 1 dyn/cm^2; > 10 dyn/cm^2) and physiological (< 10 dyn/cm^2) mechanical forces.

Physiological mechanical loading enhanced chondrogenesis with reduced apoptosis and gene expression for matrix-degrading enzymes e.g., MMP13, compared with the pathophysiological control. Mimicking the pathophysiology of OA, we stimulated the 3D cartilage model with 100 ng/mL TNF-α for 6 hours under physiological and pathophysiological mechanical shear forces. In comparison, TNF-α stimulation using a physiological shear force set-up showed reduced IL6 gene expression, soluble IL-6 amounts, and reduced expression of matrix-degrading enzymes but enhanced anabolic chondrogenic matrix proteins compared to the pathophysiological set-up.

We conclude from these data that physiological shear stress seems protective against inflammation-mediated cartilage degradation. Thus, studying the effects of mechanical stimuli *in vitro* in a highly controlled and reproducible fashion will enhance our understanding of the initial key events of this devastating disease.

Presentation: Oral

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Advancing higher education on AOPs for the integration of NAMs in testing and assessment

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Adverse Outcome Pathways (AOPs) and Mode of Action (MoA) integrate mechanistic information from a broad range of sources/lines of evidence including computational predictive models, data generated *in vitro* by new approach methodologies (NAMs), *in vivo* regulatory toxicological studies and epidemiological investigations. Integration of information along these described biological pathways is critical in informing plausibility, causality, and human relevance as a basis for predictive inference in more efficient testing and assessment strategies.

These pathway constructs are well-established in the field and widely accepted and applied by national regulatory Agencies, worldwide. Considerable experience in their assessment and application internationally has informed current guidance on best practices for developing, documenting and assessing causality in an Organization for Economic Cooperation and Development (OECD) AOP development program and associated knowledge base which includes over 400 AOPs in various stages of completion (https://aopwiki.org/).

Having been introduced in the mid to late 1990's, there is also considerable experience in the provision of training for the research community developing pathway descriptions and the application community assessing confidence in their supporting evidence bases for a range of testing and assessment purposes. Lately, core educational materials developed originally for MOA for the World Health Organization (WHO) and more recently, for AOPs within the OECD program have been expanded and evolved in higher education programs. The status of existing experience in this area will be addressed and examples of evolving curricula provided.

Presentation: Oral

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Evaluation of local tolerance of vaginal formulations and medical devices with a 3D human vaginal model

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This study validates the utility of the 3D human *in vitro* vaginal tissue model as an alternate for the rabbit vaginal irritation (RVI) assay requested for the regulatory evaluation of medical devices in contact with the vaginal mucosa. A study was conducted *in vitro* and *in vivo* with N = 14 coded test articles (TAs) known to be in contact with vaginal tissues in the form of preservatives, contraceptives, solvents, enhancers, antiseptics, and surfactants. The TAs were topically applied *in vitro* and *in vivo* at 2% dose with 5 repeat exposures over 6 days. While the RVI score was used to monitor *in vivo* irritation; MTT, TEER, and histology were used as *in vivo* endpoints. The results showed that four TA including two known irritants, benzalkonium chloride (BZK) and nonoxynol 9 (N9) were predicted as irritants by MTT viability and TEER (< 50% reduction). While BZK was identified as a mild/severe irritant in the RVI assay, the effect of N9 in rabbits was highly variable. Based on the rabbit data, we further examined the predictivity of the *in vitro* assay using EpiVaginal tissue reconstructed from cells from four donors and tested 55 TAs commonly used in feminine care products. The results showed 5 of the 55 test articles resulted in < 50% tissue viability and TEER values compared to controls. In conclusion, the use of *in vitro* EpiVaginal tissue can help reduce the need for animal testing and provide more accurate and predictive data for assessing the irritation potential of substances on human tissue.

Presentation: Oral

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Regulatory opportunities and challenges to reduce testing on animals in a next-generation risk assessment

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As pesticide regulators aim to fulfil their commitment to applying international principles on the judicious use of animals in toxicity testing, they must ensure that regulatory decisions can be reached with confidence while allowing for the reduction, refinement, or replacement of animal studies. With ongoing advances in the field of new approach methods and growing collaboration among international partners, these opportunities are presenting themselves more frequently. The review and application of alternative methodologies may help to increase regulatory confidence in these non-traditional approaches, particularly if access to alternatives coincides with the continued availability of traditional toxicity tests to allow for a comparative impact assessment on regulatory decisions. At the same time, there is a myriad of varied and competing challenges that regulators face when attempting to modernize the toxicity testing paradigm for agrochemicals. This talk will outline the opportunities and challenges facing pesticide regulators in the move toward a next-generation risk assessment and will highlight key considerations impacting pesticide regulators as they navigate transformation. By sharing these perspectives, the aim is to facilitate an understanding of the concerns facing regulators, which is an essential step in making progress toward the timely implementation of alternative approaches.

Presentation: Oral

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We need to have a respectful conversation about animal use in research

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The overall purpose of this science communication project is to increase knowledge and use of non-animal based methods. By using real stories and experiences in a documentary film production, we want to contribute to a respectful conversation about animal use in research, and by that create a debate that inspires and encourages rather than polarizes.

"The Best Model" is a documentary short film following a group of international toxicology students at Karolinska Institutet. During their studies, the students' concept of animal use in research and the relevance of animal testing for the future, are challenged. The students discuss the importance of the 3Rs (replace, reduce, refine). They talk about refinement and positive effects on animal welfare and scientific quality. Some of the students believe that animal testing is difficult to replace. Others believe that computer simulations and cell models should be able to completely replace animal testing.

The film material is used in screenings and workshops with different target groups in academia and authorities and in teaching for high school and university students. These screenings and workshops aim to engage and entertain, and to create positive change. Real stories – Real change.

Scientific development, awareness, and how we talk about animal use in research are the main themes in "The best model". This documentary film contributes to reducing prejudices about animal use in research and testing as well as prejudices about non-animal-based models. We need to have a respectful conversation about animal use in research to embrace future development.

Presentation: Oral

569

Lessons learned on validation, acceptance and uptake of the rainbow trout gill cell-based assay (RTgill-W1)

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The RTgill-W1 cell line assay to predict acute fish toxicity is the first cell line-based *in vitro* assay to gain global acceptance for chemical and water sample testing in environmental risk assessment. It has been adopted by ISO (Standard 21115: Water quality – Determination of acute toxicity of water samples and chemicals to a fish gill cell line (RTgill-W1)) and by OECD (Test Guideline 249: Fish Cell Line Acute Toxicity – The RTgill-W1 cell line assay). Systematic development, thorough standardization and validation provided the basis for this success. As the assay stands, impact on cell viability is measured based on three fluorescent indicator dyes after 24 h exposure in a 24-well plate for six sample concentrations. Fluorescent measurements are used to derive effective con-

centrations (EC50). The lowest of the EC50 values is used to directly predict the lethal concentration (LC50) in fish. In response to a surge of requests for sample analyses using this assay, we founded the aQuaTox-Solutions GmbH. Although a 1:1 replacement of the acute fish toxicity test (OECD TG203) has not been expressly accepted by regulatory agencies, chemical industry from a variety of branches as well as environmental protection agencies trust in the value of the RTgill-W1 cell line assay. Indeed, based on the services provided by aQuaTox-Solutions in 2022 alone, thousands of fish were spared, in addition to other important benefits, namely high testing throughput and little amount of test material needed. Hence, RTgill-W1 serves as model to develop further 3R-spirited cell-based assays for environmental risk assessment.

Presentation: Oral

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Confronting animal methods bias in scientific publishing

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Animal experiments are subject to high variability, do not reliably predict clinical outcomes, and are ethically problematic. Despite the availability of more reliable, effective, and ethical nonanimal experimental systems, animal use remains the "gold standard" in biomedical research and testing due to institutional inertia, financial interests, and other barriers to change. While systemic in nature, these barriers are carried out at an individual level, such as through biased manuscript peer reviews, often involving reviewers requesting that authors perform animal experiments to validate their findings. Animal methods bias in scientific publishing is a newly defined type of publishing bias describing a preference for animal-based methods where they may not be necessary or where nonanimal-based methods may already be suitable, which impacts the likelihood or timeliness of a manuscript being accepted for publication. This presentation will (1) provide evidence of animal methods bias in publishing, including anecdotal accounts and survey results, (2) explore its consequences, including the conduct of unnecessary animal experiments and negative career repercussions, and (3) discuss ongoing work of the Animal Methods Bias Taskforce to systematically gather more evidence and further mitigate animal methods bias, including providing resources and community for authors and engaging with journal editors and peer reviewers. Considering the significant impact that publishing biases may have on scientific progress and the careers of scientists, these efforts aim to contribute to the necessary shift away from animals in biomedical research and testing.

Presentation: Oral

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Introducing the Three Rs into primary and secondary schools in Europe

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Schools are the place to teach future change-makers problem-solving skills, introducing them to current issues and make them think of better solutions. Challenging the use of animals in research is key nowadays and an excellent topic to promote ethics and critical thinking in education. The European Commission Joint-Research Centre (JRC) project, coordinated by European Schoolnet (Network of 34 Ministries of Education) on the Three Rs in education, has built learning activities and introduce the principles of Replacement, Reduction and Refinement of animal experiments. Students were offered lessons introducing the topic and had an opportunity to raise their awareness of the ethical basis to the EU's Three Rs policy, they learned about animal testing in science and new technologies helping to reduce and/or replace the use of animals. Moreover, students developed critical thinking, to research different methods and innovation opportunities as well as learned about careers and jobs in the Three Rs.

The Project also targeted teachers, who had various opportunities to learn about the Three Rs, to develop their own educational materials as well as to use the materials, developed by their peers. The project fostered exchange of information, knowledge, and best practices between teachers, providing tools for education and training related to the Three Rs.

During the presentation, participants will learn more about the Project educational activities which reached over 1,000 teachers and more than 12,000 students and the results achieved.

Presentation: Oral

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Increasing scientific confidence through good practice in the application of mechanistic data

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Mode of Action (MoA) and Adverse Outcome Pathways (AOP) integrate mechanistic information from a broad range of sources in pathway descriptions of key events and key event relationships at all levels of biological organization to link exposure to specific

adverse effects. They serve as organizational and integrating constructs for relevant data from a range of sources including computational predictive models, data generated *in vitro* and by NAMs, *in vivo* regulatory toxicological studies and epidemiological investigations. The integration of information along the biological pathway, in turn, provides important information regarding plausibility, causality and human relevance.

Key Characteristics (KCs) introduced more recently, are generalized categories, or characteristics, between chemical and biological properties and human disease outcome (e.g., cancer, reproductive effects) based on empirical associations. They are not characterized by levels of biological organization as a basis to support consideration of causality but rather can be considered as potential indicators of general activity which could be associated with a disease outcome.

A series of recent and proposed workshops addresses the interface between KCs and mechanistic pathway descriptions (AOPs and MOA) to identify commonalities and potential for complementary application. Informed by different communities, these constructs have collective potential to increase confidence to support the application of mechanistic data including NAMs in hazard assessment. The presentation summarizes concepts, introduces evolving understanding and invites future collaboration to contribute to the development of good practice in the use of mechanistic data in hazard assessment to facilitate the application of NAMs.

Presentation: Oral

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Milestone based handling reduces stress and facilitates animal-experimenter interaction

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Laboratory animals are subjected to various manipulations during their time in laboratories, with scientists and animal care providers. These manipulations cause involuntary and uncontrolled stress which can have profound effects on the welfare of the animal and can also be a confounding factor for experimental variables. Studies have demonstrated that mice can be habituated to manipulations using handling techniques that result in reduction of stress and improvement in ease of handler interactions. Here, we provide a detailed description and demonstration of a newly developed mouse-handling technique intended to minimize the stress experienced by the animal during handler interactions. This manual technique makes use of milestones during handling habituation to assess an animals' response to handling and is usually accomplished over three days (3D-handling). We compare this technique to previously established tunnel handling technique (involving a 10-day habituation protocol and a PVC tunnel) and tail picking technique, in their effects on anxiety-like behaviors, using behavioral tests, voluntary interaction with experimenters and physiological measurement. The 3D-handling technique and the tunnel handling technique were found to reduce anxiety in behavioral tests and corticosterone levels, when compared to tail handling. As expected, only the 3D-handling technique improved experimenter interaction, facilitating interaction with experimenters and animal care providers. In conclusion, the 3D-handling is a useful approach to reduce mouse reactivity, improve interaction with human experimenters and care takers, reduce data variability and overall increase animal welfare.

Presentation: Oral

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Challenging the old ways: A call to rethink behavioral methods

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While conducting a systematic review of one of the most popular behavioral tests for assessing depression in laboratory rats, we came to recognize that tradition might be working against us. Researchers, in the spirit of getting reproducible results, have opted to follow methods that have remained mostly unchanged over the years. Some of these methods, initially developed with practicality and ease-of-use in mind, may have prioritized convenience over solid biological grounds. As a result, these methods might confound the results of experiments by introducing critical, and easily overlooked, methodological biases. In our systematic review, we found that such faithful compliance to tradition led to a decrease in the reliability of the test and undermined confidence in the body of evidence derived from studies using it. We would like to bring awareness to the detrimental effect that abiding to tradition might

have on the quality of animal- based research. It is a call to go back to basics, to rethink and refine behavioral methods, because if tradition comes at the cost of validity, maybe it is time to challenge tradition.

Presentation: Oral

582

The progression of MPS utilization in drug discovery

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Within the Discovery/pre-clinical phase of R&D in the pharmaceutical sector the levers for uptake of New Approach Methodologies (NAMs) differ depending on the specific context of use. The understanding of human disease, identification of targets, pathways, compound screening, ADME, the demonstration of pharmacology and human dose-selection bring unique challenges. The human-centric approach however pulls on the increased understanding of human genetics, quality cell and tissue access, utilization of stem cell technologies and increased development of complex *in vitro* models including organoids, organ on a chip and microphysiological systems. The use and uptake of MPS across the pharma industry will be discussed plus the opportunity when applicable to use animal MPS models. I'll report on how the pharma industry through the Innovation and Quality Microphysiological systems (IQ-MPS) affiliate is addressing the specific requirements of MPS across various organ systems and movement towards setting standards in recent workshops with the FDA and other global regulators. I'll conclude on where there remains gaps and a future outlook for NAMs in drug discovery.

Presentation: Oral

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Early innate and adaptive immune responses in human intestinal tissue slices from IBD and non-IBD patients *ex vivo*

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Dysregulated immune responses are a key feature of inflammatory bowel disease (IBD) and anti-inflammatory bacterial metabolites (e.g., butyrate) are considered as novel treatment option. Precision-cut intestinal slices (PCIS) are viable tissue sections that maintain intestinal microanatomy, including immune cells. PCIS have been used as translational 3R model in drug metabolism and toxicology studies, however data on immune reactivity is still missing. Here, we aimed to establish early innate and adaptive immune activation endpoints in PCIS from IBD and non-IBD patients and investigated the effect of butyrate treatment *ex vivo*.

PCIS, generated from ileum resections, showed typical morphology and retained viability during culture as assessed by histological imaging, LDH- and ATP-assay. After 24 h, LPS (1 µg/mL) or IL-1β (100 ng/mL) clearly upregulated IL-8 secretion (20-50-fold) in non-IBD PCIS. In contrast, IBD-PCIS showed already high baseline IL-8 secretion, which was not further upregulated by LPS or IL-1β stimulation. Yet, LPS and IL-1β increased intracellular ATP levels (3-fold) in IBD-PCIS, indicating higher metabolic activity. Stimulation with Concanavalin A (10 µg/mL) induced secretion of T-cell cytokines (e.g., IL-2, IL-17A). This induction was inhibited by butyrate treatment (0.1-10 mM) in IBD- and non-IBD PCIS.

Our data highlights disease-dependent differences in human PCIS and demonstrates for the first time *ex vivo* modulation of early immune responses in this model. Overall, PCIS represent a promising translational model to reduce animal experiments and study pathomechanisms and treatment responses directly in human tissue.

Presentation: Oral

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Toxicity by descent: Using phylogenetic relationships to predict interspecies differences in toxicity pathways

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Cross-species extrapolation is a critical component of chemical safety testing, yet, predicting the human health effects of chemicals is challenging, in part because toxicology has largely excluded evolutionary knowledge of how genes are functionally related. To address these needs, PrecisionTox was developed to better protect the health of people by establishing New Approach Methodologies for chemical safety testing using a mix of comparative genomics, metabolomics, evolutionary theory, quantitative genetics, data science, toxicology, and law. PrecisionTox employs six model species/cells, i.e., human cell lines, the embryos of zebrafish and frog, fruit flies, water fleas, worms, which together represent major branches of animal evolution and are recognized biomedical model systems. Our approach leverages the shared genetic legacy of toxicity response with other animals, including invertebrates, to uncover molecular toxicity pathways shared across the animal kingdom. We demonstrate that over 70% of gene families associated with disease/health status are shared among the greatest variety of animal species through evolution. Pathway conservation between invertebrates and humans is based on the degree of conservation within vertebrates and the number of interacting genes within the human network. Human gene sets that already serve as biomarkers are enriched by evolutionarily conserved genes across the animal phylogeny. These discoveries are foundational to interpreting results of biomolecular data generated by chemical screens. PrecisionTox is strengthened through the ASPIS Consortium via integration with the ONTOX and RISK-HUNT3R projects, which are developing state of the toxicokinetic modeling and next generation risk assessment approaches. Presented on behalf of the PrecisionTox project (https://precisiontox.org/).

Presentation: Oral

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Human neuromuscular junction-on-a-chip platform as a drug development tool supporting development of therapies

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Currently available animal models fail to reproduce the pathological complexities of most neuromuscular diseases, including Myasthenia gravis (gMG), a rare autoimmune disease driven by autoantibodies targeting components of the neuromuscular junction (NMJ). For the successful development of new therapies, robust alternative approaches methods (NAMs) that specifically mimic the human NMJ function are required to elucidate the toxicity mechanism of autoantibodies in gMG. In this study, the Ananda Devices microfluidic platform NeuroMuscle™ was used to create an *in vitro* 3D co-culture of motor neurospheres derived from human induced pluripotent cells and primary human skeletal muscle fibers that reproduces a functional NMJ. The platform was validated for robust detection of muscle contraction after stimulation with acetylcholine; reproducible detection of NMJ function by quantifying muscle contraction after neuronal stimulation with glutamate and rapid detection of NMJ function inhibition by alpha-bungarotoxin and D-tubocurarine. In addition, Ananda Devices' NeuroMuscle™ platform is scalable, compatible with standard laboratory equipment and uses human derived reagents and cells. Functional connectivity was assessed with glutamate stimulation of neurospheres and subsequent calcium transients in GCaMP6-transduced muscle fibres. AChR antagonists also confirmed functional connections of NMJ co-cultures developed in the NeuroMuscle™ platform. Next, the NMJ cultures in the NeuroMuscle™ platform were incubated with sera from healthy and gMG patients which induced complement activation and impaired neurotransmission. The data highlight how Ananda Devices' human based, *in vitro*, NeuroMuscle™ platform could support drug discovery in NMJ-related diseases and could be used to test compounds to understand efficacy and mechanistic rationale.

Presentation: Oral

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Comparison of dose-response modeling pipelines for developmental neurotoxicity (DNT) new approach methods (NAMs)

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A battery of developmental neurotoxicity (DNT) new approach methodologies (NAMs) has been developed to evaluate key events in neurodevelopment, including proliferation, differentiation, apoptosis, neurite outgrowth, synaptogenesis, and neural network formation. The integration of *in vitro* assays, representing diverse neurodevelopmental processes, will be a critical next step for evaluating chemicals with DNT hazard potential. The DNT NAMs battery includes assays from different experimental platforms (e.g., microelectrode array and high-content imaging) or laboratories (e.g., US EPA and the EU) that use different cell types and species. Additionally, assay developers rely on different dose-response modeling pipelines, such as the ToxCast Pipeline, CRStats, DNT-DIVER, or PROAST, to estimate efficacy and potency. In this work, we evaluated the influence of different pipelines on model outcomes, including discrepancies in cutoff thresholds, confidence intervals, points of departure, and resultant bioactivity profiles. Moreover, classification models to compute "selective" bioactivity (activity below the threshold of cytotoxicity) were compared. Understanding strengths and limitations of dose-response modeling pipelines will be integral for informing biological interpretation of DNT NAMs and application for decision-making in the regulatory process for DNT.

This abstract does not necessarily reflect Agency policy.

Presentation: Oral

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Organoid Intelligence (OI): The new frontier in biocomputing and intelligence-in-a-dish

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Recent advances in brain organoids promise to replicate critical aspects of learning and memory *in vitro*. Coining the term Organoid Intelligence (OI) to encompass these technical developments, we are showcasing the new multi-disciplinary scientific and engineering field of OI We define OI as a new frontier of a biocomputing. As such, we provide a vision for its development over the coming decade and highlight the important societal and ethical considerations it entails. We delineate its principles and potential benefits over computational AI by using biological learning to vastly improve the speed, quality and energy efficiency of computing for the benefit of science and society. Here, we describe the scientific and technological basis of OI – bringing to the fore the latest collaborative brain cell culture/bioengineering advances, providing the foundation for the new OI paradigm (allowing production of myelinated brain organoids encapsulated in to the multielectrode cages, EEG). We share a comprehensive vision of a multidisciplinary research and development trajectory that aims to further scale up the production of organoids housed in novel electrode arrays. We present the challenges and nascent solutions being developed with the potential to pioneer novel biocomputing models via stimulus-response training and organoid-computer interfaces – assessing the true learning potential of OI We delineate the necessary roles of the various disciplines involved in this inherently multidisciplinary new field – including electrophysiology, bioengineering, brain modelling, AI/big data, and bioethics. OI application goes beyond modeling of learning and memory to biological and hybrid computing as well as disease modeling.

Presentation: Oral

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GeneTox21 – An integrated platform for *in vitro* genetic toxicity assessment of new and existing substances

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Genetic damage is linked to numerous human diseases, and chemical screening programs routinely include genetic toxicity assessment. Some traditional *in-vitro* genetic toxicity tests are labor-intensive; new approach methodologies are required to efficiently assess chemicals in commerce and reduce the reliance on experimental animals. GeneTox21 is an *in-vitro* genotoxicity assessment platform that includes six complementary assays (MicroFlow®, MultiFlow™, CometChip®, TGx-DDI biomarker, MutaMouse FE1 cell mutagenicity, and Ames II bacterial mutagenicity) that comprise a wide range of endpoints measuring DNA damage, mutation, and chromosomal abnormalities. Platform performance is being evaluated using 35 reference compounds and 20 data-poor substances flagged using structure-activity screening. In addition to positive or negative classification, the Benchmark Concentration (BMC) approach is being employed to quantitatively determine substance potency. Initial results indicate that the platform readily detects genotoxicants, including those that require bioactivation for generation of DNA-reactive metabolites. Multiplexed response profiles reflect the substance's MOA (mode-of-action), e.g., mutation, chromosomal damage, aneugenicity. Analysis of data-poor substances shows that SAR predictions frequently differ from actual genetic toxicity profiles; multiplexed responses provide essential information on relative potency and MOA. The Integrated Analysis Tool for Genotoxicity Assessment (IATGA) was developed to permit streamlined visualization, quantitative analysis, and regulatory interpretation of GeneTox21 data. IATGA permits IVIVE (*in vitro*-to-*in vivo* extrapolation) to determine Administered Equivalent Dose (AED) values. Empirical comparisons of BMC and AED values suggest that *in vitro*-derived point-of-departure metrics are conservative. The GeneTox21 platform allows for comprehensive, multiplexed assessments of chemically induced genotoxicity; IATGA permits data analysis and interpretation for screening and prioritization.

Presentation: Oral

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Modeling life stage toxicokinetic variability for effective chemical prioritization

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An underlying need in chemical risk assessment is to ensure that sensitive populations are identified and adequately protected. Bottom-up approaches using *in vitro* or *in silico* data and physiologically based kinetic (PBK) modeling can quantitatively estimate chemical-specific toxicokinetic (TK) variability, identifying both the extent of variability present and key drivers. Here, an *in vitro-in vivo* extrapolation (IVIVE) PBK modeling approach is being utilized to estimate steady state concentrations (Css) for hundreds of commercial chemicals of importance to the US EPA. Employing isozyme-level xenobiotic metabolism predictions anchored to experimental measures of hepatocyte clearance, Css outputs are predicted across a range of life stages and populations to derive human toxicokinetic adjustment factors (HKAFs), quantitated by dividing 95th percentiles by median values of a healthy adult population. To date HKAFs have been estimated for twenty compounds, selected to capture a range of compound types (acids, bases, neutrals), TK, and Cytochrome P450 (CYP) metabolism. Highest HKafs were consistently returned for two-week-old infants, with a maximal value exceeding 9 returned for triclosan. Higher values were observed in a CYP-specific manner, exhibiting the following rank order: CYP1A2 > CYP3A4 > CYP2C19 > CYP2C9 > CYP2D6. Also, higher values were observed for those chemicals that were substrates for no more than two isozymes. Evaluations across more chemicals, subpopulations, and other aspects of TK and genetics (i.e., polymorphisms) are planned. Ultimately, PBK modeling and simulations are uniquely positioned to identify priority research needs and to aid in transitioning beyond default uncertainty factors.

This abstract does not necessarily reflect the views of the U.S. EPA.

Presentation: Oral

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Animal-free science education and training in Brazil and LATAM

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The Law 11,794/2008 was a milestone for the regulation of activities related to the use of animals in scientific experimentation and education in Brazil/LATAM. This law supported the creation of the National Council for the Control of Animal Experimentation, for monitoring and introduce alternative to animal testing. In 2011, we hosted the first International hands-on workshop of alternative methods in Brazil. The Brazilian National Network of Alternative Methods (RENAMA), established in 2012, is currently composed of 51 laboratories from CROs, academia, industry, and government. The RENAMA proposes the development and training/education on alternative methods, reducing LATAM dependence on external technology. In 2015, the Regional Platform of Alternative Methods to the Use of Animals (PReMASUR) was created to run the education and training activities, including Argentina, Brazil, Paraguay and Uruguay. Here, we present the example of the Laboratory of Education and Research in *In vitro* Toxicology – FF/UFG, Brazil, member of RENAMA/PReMASUR, which since 2004 has introduced new *in vitro* scientific concepts to undergraduate and graduate students of innovation in Pharmaceutical Sciences. Over 110 undergraduates and more than 45 MSc/PhD students per year are introduced to new paradigms in Toxicology for a new Science based on human biology, without suffering and pain to animals. Professionals, scientists and regulators from Brazil and LATAM were also trained in NAMs by our team (in-person lectures, networking opportunities, online webinars, hands-on demos). The current paradigm is ingrained in students and education/training is the basis for a shift in thinking beyond animal models.

Presentation: Oral

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Accelerating animal free-innovations: Milestones and lessons learned in the Dutch transition program

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The transition from animal experiments to non-animal methods (NAMs) is an exceptionally complex challenge that needs commitment from various stakeholders. The Dutch Transition partner program to accelerate animal-free Innovation (TPI) was founded in 2018 in conjunction with Replacement, Reduction and Refinement policy, a European policy that is still in place. TPI forms a unique collaboration, as it is seldom shown that a national government – in this case the Dutch Minister of Agriculture, Nature and Food quality – directs such a program with external partners.

The TPI program consists of eleven partners, who jointly give practical substance to the program. Recently the partners have adopted "Better predictions without lab animals" as their mission. During the past four years, we achieved various milestones. With these experiences in mind, the Netherlands, with TPI, wants to become a catalyst country in the (inter)national transition towards NAMs.

Key elements that bind partners to TPI are its multidisciplinary network, knowledge sharing, the positive directed mission and the acknowledgement of each other's differences. A transition requires changes at various fields and levels, therefore innovation as well as collaboration between stakeholders (e.g. validation roadmap and knowledge agenda), involving early-career students and researchers (Young TPI), communication and adaptions in education are vital. Furthermore, acceptance of NAMs need to be achieved by European and worldwide adoption. Hence our ambition to act as a catalyst for the development of NAMs. However, we will not realise that ambition succesfully if partnerships are missing. This is why we reach out for you.

Presentation: Oral

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The SARA-ICE model for predicting skin sensitizer potency

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The Skin Allergy Risk Assessment-Integrated Chemical Environment (SARA-ICE) model is a probabilistic defined approach (DA) that provides a weight-of-evidence point of departure (PoD) and GHS potency prediction for use in skin sensitization assessments. SARA-ICE is constructed within the Bayesian statistical framework using data sourced from the Integrated Chemical Environment (https://ice.ntp.niehs.nih.gov/), Unilever SARA publications, and Cosmetics Europe. SARA-ICE predicts a human relevant PoD: the ED01, the dose with a 1% chance of inducing sensitization in a human predictive patch test (HPPT). The PoD can be cal-

culated using data from HPPTs, local lymph node assays (LLNA), and new approach methodologies (NAMs). For a chemical of interest, the model returns the probability of each GHS classification conditional on the distribution of the ED01. We used the OECD DA for Skin Sensitisation (TG 497) reference data set to evaluate SARA-ICE for GHS classification accuracy. Using a probability of 0.8 as the binary classification criterion, balanced accuracy was 97% for conclusive calls relative to human classifications, but 36% of chemicals had inconclusive calls. Using a probability of 0.55 for the subcategory classification criterion, average balanced accuracy was 85% for conclusive calls versus human classifications. A case study on isothiazolinones demonstrated that SARA-ICE performed well, correctly identifying these broad-spectrum preservatives as sensitizers. SARA-ICE will be made freely available online, enabling users worldwide to easily predict human skin sensitization potency without animal testing and supporting probabilistic risk assessment applications.

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Presentation: Oral

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Virtual human platform for safety assessment (VHP4Safety)

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The VHP4Safety project aims to protect human health and improve the safety assessment of chemicals and pharmaceuticals without the use of animals. Three case studies are designed to incorporate human relevant scenarios to discriminate vulnerable groups that are not addressed in animal studies. These include disease state (kidney failure) upon exposure to pharmaceuticals, life course exposure to pesticides and neurodegeneration and differences in sex and age upon exposure to chemical substances in the development of thyroid-mediated brain development. Several data sources, e.g. data on human physiology, chemical characteristics, existing (omics) data, epidemiology data, toxicokinetic and -dynamic parameters, are integrated within Adverse Outcome Pathway (AOP) networks together with data generated from *in vitro* assays representing key events in the AOPs. These data feed into the VHP4Safety *in silico* platform built on tools and services such as PBPK, machine learning and knowledge driven analysis. We will present the contours of the VHP4Safety platform, case study workflows that feed the platform and activities towards the implementation of the platform, such as setting performance criteria, regulatory acceptance and education and training. Through active involvement of academic, regulatory, industrial and societal partners covering the entire innovation chain, we are developing a new framework to determine the safety of chemicals and pharmaceuticals to protect human health solely based on human biology. While working together with other initiatives, like the European projects of the ASPIS cluster, VHP4Safety will accelerate the transition to animal-free safety assessment.

This project (www.vhp4safety.nl) is funded by Dutch Research Council (NWO): NWA-ORC 1292.19.272.

Presentation: Oral

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Dissemination of science: Animal-Free Safety Assessment (AFSA) education and training modules

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Significant progress has been made globally over recent years in advancing the science to underpin non-animal cosmetic safety assessment. In addition, restrictions on animal testing of cosmetics and cosmetic ingredients are expanding geographically every year. These advances are leading to the need for building capability and capacity for completely animal-free safety assessment of consumer products amongst the regulated and regulatory communities.

Achieving a confident risk assessment of a consumer product or ingredient without data from new animal testing requires a novel approach to the assessment as well as integration of several types of *in silico* and *in vitro* data. It is important to build this confidence in the requisite methodologies based on experience. Toward this aim, an in-depth educational program has been developed by the AFSA (Animal-Free Safety Assessment) Collaboration, an ambitious partnership of NGOs and industry. The course covers the risk assessment process from beginning to end in 8 modules: Problem formulation, Consumer Exposure, Exposure-based Waiving, History of Safe Use, *In silico* Tools & Read-Across, Internal Exposure: Dosimetry, *In vitro* Data Synthesis, and the Overall Risk Assessment. A ninth module covers the global regulatory landscape for cosmetics and chemicals. Here we present short overviews of each module; the entire course is available as a free, online self-paced course (www.AFSAcollaboration.org).

Presentation: Oral

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Accelerating the transition to animal-free safety assessment: What can we learn from the cosmetics animal testing bans?

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Animal-free safety science has advanced a great deal in the twenty first century. Investment in New Approach Methodologies (NAMs) and Next Generation Risk Assessment (NGRA) approaches have enabled significant progress in replacing regulatory animal tests and a global paradigm shift in chemical regulatory testing is now well underway, catalysed by a succession of cosmetic product and ingredient animal testing bans.

We have collectively learnt that combining NAM data with exposure information using computational approaches enables us to set, and assess against, more meaningful human and environmental protection goals. Consequently, it has become clear that transitioning to animal-free NAMs and NGRA approaches can help us better protect people and our planet, support sustainable chemical innovation, and replace animal testing.

So where are we today? After two decades of rapid progress, we find ourselves at a tipping point as we collectively transition from early adoption to widespread use of NAMs and NGRA for chemical safety assessment. This talk will review what we have learnt from widespread Cosmetics Industry adoption of animal-free safety science, discuss the current global state-of-the-science for use of NAMs and NGRA approaches for Chemical Safety Assessment, and share a perspective on how we can accelerate the transition through a renewed focus on education and training.

Presentation: Oral

606

Advancing Three Rs uptake in university education through a European network

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The new advanced methodologies in Three Rs Education in Universities, require a mandatory integrated teaching strategy, with a multistep vision, from bachelor to master's degree to PhD, coordinated by a European network. A sort of a dynamic bottom-up approach, re-enforcing during the curricula the knowledge on NAMs and Replacement, through a harmonized European perspective, to find a new common and coherent direction to implement courses in academia to form future European aware experts not only in science, but in all the activities intertwined to the 3Rs, like communication, ethics, innovation, policy, regulation and others.

A first basic and unique 3R course will have to be expected in the first year of all university curricula both related to Life Sciences (LS) and Human/Social Sciences (HSS), followed by a second dedicated course at the third year of LS curricula, i.e. Animal Science, Biology, Biotechnology, Chemistry, Environmental Sciences, Medicine, Physics, Veterinary Medicine etc. and HSS curricula i.e. Economy, Law, Philosophy, Political Sciences etc. Two specific master's degree on 3Rs (2 years) could be hypothesized for scientific career in LS, and another one in humanities. PhD courses will have to include a didactic activity of minimum 4 to 20 hours per year on 3Rs, in relation to the discipline. The harmonizing role of the European network on Three Rs Education in university between the different national realities, will work respecting and emphasizing the aspect of multidisciplinarity, interdisciplinarity and intersectionality.

Presentation: Oral

609

Enhanced caging standards for rats: Effects on welfare and historical data

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Recently published guidelines from the Canadian Council on Animal Care (CCAC) mandate that laboratory rats be housed in cages that allow them to stand upright and perform vertical stretches; behaviours that are restricted by the height of currently used standard cages. Taller cages may improve rat welfare but could influence historical scientific data and impose operational constraints on staff. To assess the effects of taller cages, forty-eight Sprague-Dawley rats (24 female and 24 male) were housed for 3 months in either standard (20 cm tall) or taller cages (32 cm tall); taller cages also contained a loft. There were no differences in anhedonia ($F_{1,12} = 0.06$, $p = 0.81$), weight gain ($F_{1,12} = 0.42$, $p = 0.53$) or glucose levels ($F_{1,12} = 0.89$, $p = 0.37$); female rats in taller cages showed higher red blood cell counts than female rats in standard cages ($p < 0.05$). The time it took staff to weigh animals and perform cage changes was similar between cage types. Staff found the taller cages to be heavier and more difficult to handle than the standard cages, but most were pleased with the perceived beneficial effect of the taller cages on rat welfare. Our results indicate that the transition to a taller cage that meets the CCAC guidelines is fea-

sible without compromising historical scientific data, and despite requiring more effort, staff are generally satisfied with the welfare benefits provided by these taller cages.

Presentation: Oral

610

PRIVAT: A peer review tool for appraising *in vitro* studies

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With the increasing use of *in vitro* systems for risk assessment – as well as basic and translational research – comes the increasing submission of manuscripts describing *in vitro* experiments to a wide array of journals and the need for peer reviewers to have sufficient expertise to properly assess these studies for scientific publication. However, peer review of *in vitro* studies faces some challenges, and the current process is not consistently effective when it comes to ensuring published studies are sufficiently scientifically robust. We have developed a Peer-Review *In vitro* Appraisal Tool: PRIVAT. PRIVAT provides a structured process for making peer review of *in vitro* studies more comprehensive, useful, and transparent. We describe the development of PRIVAT, its capabilities, and how researchers can use the tool to improve the quality and community value of their peer-review comments. It is one contribution of the Evidence-based Toxicology Collaboration to improve manuscript peer-review and, ultimately, the publishing processes in toxicology.

Presentation: Oral

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The effect of environmental enrichment on anxiety-like responses of mice: A systematic review

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Laboratory mice are commonly housed in shoebox cages; this housing is associated with restricted expression of natural behaviour and a high incidence of abnormal behaviour. More enriched environments can promote positive and reduce negative affective states (e.g., anxiety, depression, boredom), but variability across studies in the type of environmental enrichment used, duration of exposure and other methodological aspects, make it difficult to develop recommendations. To better understand the available evidence, we reviewed environmental enrichment studies that assessed anxiety-like behavioural responses in the elevated plus maze and open field test. We used a literature search of MEDLINE and Web of Science and retained 25 out of 186 studies for data extraction and estimation of the standardized mean differences between intervention and control conditions. In the elevated plus maze, we found that 50% of enrichment interventions had a medium to large positive effect in the time spent in the open arms (i.e., decreased anxiety). Differences were noted between strains and sex, with larger effects on C57BL/6J than BALB/c and males than females. In the open field test, we found that 30% of the interventions had a medium to large positive effect in the time spent in the center of the arena (i.e., decreased anxiety); but open field-testing arena size and test time were highly variable across studies. We conclude that enrichment interventions, especially those that used a combination of features, including running wheels, ladders, tubes, and shelters, consistently reduced anxiety-like responses in mice.

Presentation: Oral

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The three pillars of ethical research with nonhuman primates

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This talk provides an overview of a novel "Three Pillars" approach to research with nonhuman primates: Harmonization, Replacement, and Justice. Harmonization entails protecting all primates (human and nonhuman) similarly by harmonizing research ethics, regulations, and guidelines, particularly those focused on children and incarcerated or institutionalized humans. Replacement builds on the goals stated in the 3 Rs and calls for the replacement of nonhuman primates in research with human-biology-based methods, rather than simply shifting research onto other nonhuman animals. Justice models the approach used in the Belmont Report and defines justice as the fair selection of research subjects: based on scientific necessity rather than on convenience. The focus on harmful research involving captive nonhuman primates is motivated in part by trends that show increasing use within several areas of harmful research, including neuroscience and vaccine development, and by efforts within the scientific community to address a so-called shortage of nonhuman primates through the creation of a "strategic monkey reserve" and demands for additional government funding for breeding facilities. The Three Pillars approach provides a path forward and away from the use of nonhuman primates in harmful research.

Presentation: Oral

613

OECD validation of the ToxTracker assay for genotoxic mode of action assessment

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ToxTracker is a mammalian stem cell-based reporter assay that detects activation of specific cellular signaling pathways upon chemical exposure. 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. More recently, the assay was extended to allow the discrimination between clastogenic and aneugenic compounds.

The ToxTracker assay was evaluated in a large international inter-laboratory validation study, approved by the OECD. The goal of this prospective validation study 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. The validation has been conducted strictly following OECD guidance document 34.

ToxTracker was transferred to seven laboratories. The validation labs were trained to perform the assay and tested a training set of compounds to show their proficiency to run ToxTracker. Next, the labs evaluated a selection of 64 coded, well-established genotoxic and non-genotoxic chemicals with each compound being tested in three labs independently. The accuracy to predict genotoxicity was 89%. Also, the intra- and inter-laboratory reproducibility were determined. The mechanistic information that was provided by ToxTracker was used to gain insight into the MoA of genotoxic compounds and to explain positive results from the standard *in vitro* genotoxicity assays.

Presentation: Oral

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Linking exposure to effect: The role of toxicokinetics in ASPIS

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One key aspect of the next generation risk assessment approach is the comparison of external human exposure to the hazard values derived from relevant new approach methods (NAMs). In the ASPIS cluster, RiskHunt3R, OnTox and PrecisionTox are developing complementary *in vitro* as well as *in silico* tools and approaches to characterize the absorption, distribution, metabolism and elimination (ADME) of the compounds. The ASPIS kinetic working group aims to integrate these approaches into ASPA (ASPIS Safety Profiling Algorithm) based on a well-defined problem formulation. This talk introduces the current modular assessment elements contributing to the ASPA kinetic framework with regard to the assessment of aggregated exposure, the integration of absorption and metabolism into *in vitro* to *in vivo* extrapolation (IVIVE) and the application of *in vitro* biokinetic modelling approaches. The learning from the case studies will be used to refine and improve ASPA, in particular to define more concretely the criteria that will guide the analysis from a first to a higher tier of evaluation.

Presentation: Oral

615

Early screening using cheminformatics in an integrated assessment for neurotoxicity/ developmental neurotoxicity

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A battery of assays to screen substances for developmental neurotoxicity (DNT) potential is being developed and evaluated. This battery is based on new approach methods (NAMs) for key neurodevelopmental events (e.g., neuronal migration, neuronal differentiation), but these data should be contextualized in an "Integrated Approach to Testing and Assessment" (IATA) with additional information such as read-across/QSAR data, toxicokinetics (*in vitro*-to-*in vivo* extrapolation), and possibly alternative *in vivo* assessments before deciding whether additional mammalian toxicity studies are needed. This presentation will focus on the value of cheminformatics data to predict potential *in vitro* results and/or select relevant NAMs. A few case study chemicals will be used to illustrate the utility of mechanistic predictions (e.g., acetylcholine receptor interaction, mitochondrial inhibition). In addition, critical stages of development and gestational stage warrant consideration to better understand the sensitivity of assay endpoints and impacts on toxicokinetics. This presentation will identify some points to consider when integrating data streams to screen chemicals for DNT potential and how this may aid in subsequent decisions for follow-up DNT testing.

Presentation: Oral

619

Reagent responsibility: IACUC assessment of research tools

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Researchers are increasingly agreeing on the scientific and ethical advantages of animal-free reagents, including animal-free recombinant antibodies and chemically defined cell culture media. However, implementing a transition to these reagents is complicated due to reagent availability, time and resources involved in the use of new reagents, and the lack of widespread awareness of non-animal options. The presentation discusses the role that Institutional Animal Care and Use Committees (IACUCs) have in addressing these barriers to implementation.

The Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals require and encourage, respectively, that IACUCs consider alternatives to animal use. It is important that reagents are assessed during protocol reviews and that assessment criteria are kept updated. An increasing number of research antibodies are available animal-free or without ongoing animal use for production, and there is growing availability of animal-free cell culture media. In order to sustain and expand the availability of non-animal reagents, oversight bodies such as IACUCs have a central role in assessing the purchase and in-house development of antibodies and cell culture media, educating researchers on animal-free options, and guiding researchers to resources to successfully transition to animal-free reagents. Examples will be provided on how IACUCs can address the use of reagents in research protocols and, on a larger scale, within their institutions.

Presentation: Oral

620

Initiatives from the European Commission to promote animal-free research

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The European Commission, through its Research and Innovation Framework Programmes (FPs), has been a major supporter of alternatives to animal testing. During the last two decades, above EUR 1 billion have been dedicated to support more than 300 projects to develop a variety of human-relevant non-animal methods and strategies. Some of these tools are currently being used in the pharma industry or for regulatory purposes. The budget to this area has constantly progressed over the years. In FP5 (1998-2002), the annual budget dedicated to this area was on average around EUR 11 million. It tripled from FP5 to FP6 (2002-2006), increased by 50% from FP6 to FP7 (2007-2013) and by 60% from FP7 to Horizon 2020 (2014-2020). The annual budget for alternatives to animal testing during Horizon 2020 represents more than EUR 75 million per year on average. In addition, industrial sectors have complemented this effort by providing at least an additional EUR 150 million. Since FP7, with the establishment of the European Research Council (ERC), the number of projects on alternatives led by early career researchers has significantly increased. The further development of alternatives to animal testing is being pursued in the Horizon Europe Framework Programme for research and innovation that runs from 2021 to 2027. The 2024 Horizon Europe work programme contains several topics for the development and use of alternatives to animal testing in biomedical research or for increasing the confidence in New Approach Methodologies (NAMs).

Presentation: Oral

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Insights from the home cage: Data science-driven activity analysis enables sensitive welfare assessment in colitis models

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Welfare assessment of animals used in biomedical research is mandatory from an ethical and scientific point of view. It is well established that impaired welfare reduces activity in various species, including mice and humans. As test systems might capture different drivers of activity, they might vary in their sensitivity to reflect impaired welfare. In a mouse model of acute colitis, we utilized three different home-cage-based activity measurement techniques – voluntary wheel running behavior (VWR), telemetry, and impedance-based locomotion measurement. Data science approaches were applied to enable objective grading of welfare in these mice. Here, we compare the outcomes and bring them into a broader view of routine welfare assessment.

All three activity-based measures revealed reduced activity during colitis. Compared to model-specific clinical scoring (including stool consistency), they were equally or more sensitive to detecting impaired welfare. In the VWR approach, k-means clustering of the data enabled the assignment of mice into three distinct severity levels. Impedance-based monitoring using a digitally ventilated rack system revealed subtle changes in activity. A binary classifier enabled categorization into severity classes. Likewise, diminished activity was observed by telemetry. The assessment tool RELSA revealed activity to be one of the most valuable parameters in this model and categorized severity on a relative scale using telemetric data.

All approaches displayed here were validated in model systems. The data science tools are publicly available. Sensitivity, applicability, possibilities, and challenges associated with these methods will be discussed.

Presentation: Oral

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Academic transition lessons: Involving stakeholders, time trials, and breeding results

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Refined mouse handling (a method of picking up mice with a tunnel or cupped hands) is a well-documented refinement that demonstrably improves mouse welfare as compared to traditional methods. Although many research facilities in the UK and Europe have switched to using refined handling methods, adoption and implementation rates in North America remain low. Our objective in this project was to transition one large academic institution in the USA to switch to refined handling methods through strategic processes and conducting key internal studies.

Throughout the process, key stakeholders were involved to generate buy-in and gradually make the transition. In one internal study, we collected self-reported time required to perform routine husbandry procedures with and without refined handling methods. In an additional internal study, we performed a randomized controlled trial to examine the effects of a standard method of han-

dling (tail-lift with forceps) versus refined handling with transfer tunnels on breeding performance in 60 C57BL/6J mouse pairs.

We found that using refined handling did not increase time required to perform routine husbandry. Additionally, breeders produced significantly more mouse pups. Ultimately, as a result of strategic stakeholder involvement, these data, and projected cost savings due to production increases, we succeeded in persuading facility management to incorporate refined handling as standard of care across the institution.

Presentation: Oral

629

Achieving an animal-free product safety assessment

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Pesticides are some of the most strictly regulated and tested chemicals, requiring over 8,000 animals for human safety and another 2,000 animals for ecological safety. Syngenta is developing an approach to generate the necessary information to meet the regulatory requirements for a new pesticide registration without the use of chemical-specific vertebrate tests. A case study applying existing modern scientific conceptual frameworks was used to identify and characterize the dose-range over which potential adverse effects may occur, relative to the anticipated exposure from proposed uses of a new AI. This informs a determination on whether these frameworks meet risk assessment needs for human health and vertebrate ecotoxicology and identify where further development of new approaches for compound-specific data generation are required. We began with a case study for a new active ingredient with high read-across potential, based on membership of a well understood pesticidal MOA: e.g. an ACCase inhibitor herbicide. This demonstrated that safety data can be curated and analyzed to provide an appropriate human health and vertebrate ecological hazard characterization for the purpose of risk assessment. We further explored its possible extension to crop protection chemicals with less extensive existing exemplar datasets and highlight the identified uncertainties and gaps that will require additional new approaches. In addition to an overview of the scientific methods to address the key knowledge needs without the use of chemical-specific vertebrate tests, a possible structure for the registration package of the future will be presented.

Presentation: Oral

630

Industry implementation of refined mouse handling: A roadmap, challenges, & solutions

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Global pharmaceutical companies are often tasked to ensure procedures and processes are harmonized across the global stage to ensure results are comparable. These procedures include methods of animal handling. In the United Kingdom, refined methods for picking up mice via a tunnel or cupped hands have become widespread in animal research. In turn, our objective was to facilitate our large USA-based mouse facility to transition to these refined methods in a one-year span.

Our strategy included utilizing external experts and a gradual transition that began with husbandry and technical support staff before engaging researchers. Informal feedback and formal yearly surveys were gathered from staff.

As a result, 45% of survey respondents in 2022 report using refined handling over 90% of the time, which is an increase of 4% from 2021. Additionally, barriers were identified and solutions created to issues involving water valve flooding, obese mice, and aversion to the tunnel. Furthermore, we found that although some users do not like the tunnel, that many prefer cupping or "opportunistic" handling.

In conclusion, we found key challenges and solutions in transitioning a large pharmaceutical facility to refined mouse handling. Ultimately, a slow, strategic transition and constantly taking in feedback are strategies for the widescale practical implementation of refined mouse handling.

Presentation: Oral

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Transcriptomic points of departure for fertilized Japanese quail embryos exposed to seven pesticides using the EcoToxChip test method

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Transcriptomics dose-response analysis (TDRA) has emerged as a promising approach for integrating toxicogenomics data into risk assessments. Here we combine an early life stage (alter-

native to animal) avian embryo test and a targeted qPCR array (EcoToxChip; n = 384 genes of relevance to environmental toxicology) to determine if 1) transcriptomics points of departures (tPOD) can be derived from this test system; and 2) the tPOD values are protective of levels associated with adverse outcomes. Fertilized, unincubated Japanese quail (JQ) eggs were injected with graded concentrations (100, 32, 10, 3.2, 1, 0.32, 0.1 µg/g egg plus DMSO control) of 7 pesticides: carbofuran, chlorpyrifos, endrin, ethoprophos, glufosinate ammonium, permethrin, and trichlorfon. Phenotypic endpoints including mortality, infertility, embryo mass, and deformity (presence of fatty liver) were assessed at embryonic day 9. Gene level BMDs (benchmark dose) were derived for each chemical. While there were relatively few gene-level BMDs (< 25 per chemical), they were lower than levels associated with adverse outcomes. Further, for all test chemicals, investigation of gene- and pathway-level results concurred with expected mechanisms of action. Finally, two of the chemicals were repeated and yielded similar results, thus giving confidence to the test method. In conclusion, the EcoToxChip Test Method can yield gene level BMD values that are protective of levels associated with adverse outcomes, and also give insights into mechanisms of action. However, derivation of tPODs may be challenging with the reduced gene set provided by EcoToxChip thus necessitating more work in this area.

Presentation: Oral

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Engineering a computable epiblast for *in silico* modeling of developmental toxicity

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The developmental potential of human pluripotent stem cells (hPSCs) in culture closely resembles the "epiblast" during gastrulation. ToxCast provides *in vitro* bioactivity data on over 1000 chemicals from a hPSC assay that predicts developmental toxicity with ~80% balanced accuracy (Zurlinden et al., 2020). Computer modeling of the epiblast in 3D would parallel the utilization of ToxCast data to track cellular trajectories during simulated chemical exposure. We engineered a fully computable model of the epiblast using the CompuCell3D that simulates primitive streak formation, epithelial-mesenchymal transition of epiblast cells, and self-organization of mesodermal domains (chordamesoderm, paraxial, lateral plate, posterior/extraembryonic). Determination of progenitor cell fate is dependent upon positional information and temporal colinearity of a HOX clock regulated by a control network of morphogenetic signals (FGF, BMP, NODAL, ATRA, CDX). Executing the model renders a quantitative cell-level computation for mechanistic evaluation of mesodermal subpopulations. Consequences of perturbation were shown, for example, on posterior mesoderm cell mass that gives rise to most hemangiogenic precursors in the yolk sac blood islands. Interfering with the signaling network produces effects mirroring those reported in experimental mouse embryology, with 50% reductions in both FGF4 and BMP4 signaling resulting in 88% and 63% reductions, respectively, in the posterior mesodermal population. This cell agent-based model integrates signaling cascades, ToxCast chemical bioactivity data and known embryology to mechanistically predict altered phenotypes through the resulting mesodermal topography in the animal-free zone.

This abstract does not necessarily reflect USEPA policy.

Presentation: Oral

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Unbroken physioxic conditions for reproducible, predictive *in vitro* human models

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Nobody ever writes, "Expose cells to non-physiologic room air conditions for variable lengths of time," into their protocols, but that is exactly what is done in traditional room air labs and even cleanrooms. The replacement of animal models with New Approach Methodologies (NAMs) requires *in vitro* human-centered models that are reproducible and predictive of human physiology. Supraphysiologic room air oxygen is an invisible problem that adds redox stress to cultures and adds artifact into toxicological assays. We have developed Cytocentric technology that can maintain constant O_2, CO_2, temperature, and relative humidity around cultures at all times, even during handling. HIF-1a, upstream of signaling pathways for cell proliferation and differentiation, is a key regulator of the cellular response to oxygen changes. Here we present data showing that constant physiologic oxygen prevented HIF-1a modulation in human MSC in contrast with conventional room air incubation and handling. We found improved MSC yields, increased proliferation rates, and extended active passage numbers. Because cells and tissues have a history, cell and tissue culture conditions need to be controlled not only during the assay, but upstream as well. Controlling cell conditions to constant, physiologic levels is a practical way to improve reproducibility and predictive power for NAMs and all human cell-based tox assays.

Presentation: Oral

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Public policy: A key to driving scientific innovation and protecting animals

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Public policy efforts serve as a key component of the work that must be done to advance scientific innovation, and eliminate outdated practices, to improve human health and safety and protection of animals. This work focuses on ultimately replacing the use of animals for biomedical research and testing of products, such as pesticides, chemicals, cosmetics, and drugs, with new approach methods that are more human-relevant and proving to be more accurate than animal tests, such as microphysiological systems, organoids, *in silico* modeling and others. It is important to recognize there are also numerous barriers to progress, including existing laws, regulations, and guidance that mandate, or essentially mandate, the use of animals in the United States and worldwide. There is historical evidence of how applying various public policy approaches has driven change in the United States and how stakeholder involvement has been instrumental to these successes. Lawmakers not only pass laws but can ensure progress, accountability and continued financial support of government agencies with efforts focused on advancing new approach methods. Government agencies are critical to removing barriers and ensuring development and uptake of new technologies and approaches. A paradigm shift is needed, and it will take non-governmental organizations, the corporate sector, and the public to continue to act for meaningful change. Real-life examples of how policy efforts at the federal and state levels in the United States have advanced this paradigm shift will be discussed.

Presentation: Oral

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A roadmap from single organ models to the integrated virtual human twin in disease and toxicology

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The last decade has seen an enormous growth in the generation and translation of *in silico* medicine technologies. Computer modeling and simulation has become a routine tool in the development, de-risking and personalisation of medicinal products and the digital evidence it generates is frequent used in the regulatory dossiers. It is also increasingly finding its way into the clinical practice in the form of planning services and clinical decision support systems. However, most of the solutions that are currently commercially available are single-organ solutions, often focusing on a single spatiotemporal scale. The EDITH coordination and support action, funded by the European Commission, is proposing a roadmap to allow the development of the integrated virtual human twin. It has brought together the entire ecosystem, meaning all stakeholders (academia, industry, healthcare professionals, regulatory agencies, HTA, patients and payers) from software, hardware, devices and pharma backgrounds, to identify the research challenges and required infrastructure that will facilitate the integration of different models, algorithms and data sets across different organ systems and spatio-temporal scales. The required efforts do not pertain only to the technology (interoperability, access etc.), but also to the ethical, social and legal frameworks, as well as the stakeholder and perspectives, and the sustainability (through public and private initiatives). Given the systemic and multi-scale nature of many toxins, the virtual human twin infrastructure can become an important asset for the further development of *in silico* toxicology solutions. The roadmap can be accessed from August 2023 onwards on www.edith-csa.org.

Presentation: Oral

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Overcoming mindset as a barrier to implementing change

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Mindset has been reported to be a common barrier that needs to be overcome when it comes to delivering cultural change within the laboratory animals sciences. This is because an individual's mindset can influence their willingness to consider and accept different ways of working and their ability to implement change. It can also affect the local research culture as we are all influenced by what we hear and see going on around us. In this presentation I will discuss how awareness of mindset traits, triggers and behaviours can help individuals develop their mindset to fulfil their research potential. Educators, mentors, and supervisors can learn to identify mindset characteristics in others so that they can offer the support or guidance that individuals need to develop behavioural traits beneficial to themselves and others working around them. Research groups, institutes and organisations can use mindset awareness to enhance the local research culture, but also to inform discussions and decisions regarding how individuals' behaviours are managed or rewarded to facilitate change.

Presentation: Oral

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Development of a 3D genotoxicity model for assessment of cosmetic formulations

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A variety of *in vitro* assays exist to examine the genotoxic potential of compounds, however the application to real-life exposure is questioned. We show that 3D tissue models can be effectively combined with an *in vitro* animal-free genotoxicity screen, overcoming insoluble formulations and with dosing that mimics real-life application. This system allows investigation into whether potential genotoxic compounds can pass through a reconstructed skin barrier, and if so, remain genotoxic following exposure to skin metabolic enzymes. Furthermore, use of the animal-free BlueScreen test allows for identification of all 3 classes of genotoxins; mutagens, clastogens and aneugens.

In summary, we created a co-culture system consisting of TK6 cells and EpiDerm tissue models which was validated using a panel of known genotoxic and non-genotoxic agents. Five concentrations of each chemical were dosed onto the apical side of the tissues for 48 h. TK6 cells were collected 48 h post-dosing and quantified for genotoxicity and cytotoxicity measurements.

Results show that the co-culture system was at least 70% concordant with the results gained from a BlueScreen test and in-depth analysis of metabolic profiles between human liver S9 and the EpiDerm tissue models explained the remaining differences. This demonstrates the greater physiological relevance of incorporating skin metabolism and a functional barrier to aid to model systemic genotoxicity following skin absorption.

The test has been used to assess genotoxicity of final formulations that don't require dilution (e.g. hair dyes) mimicking real-life application to determine how the skin barrier can modulate genotoxic potential.

Presentation: Oral

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A novel welfare primate welfare assessment tool for research primates

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Primates are important species in biomedical research and therefore we have an ethical obligation to provide good welfare. Systematic animal welfare assessments are important for ensuring continuous improvement of animal care and use programs. Through a multi-facility collaboration, a Primate Welfare Assessment Tool (PWAT) was developed to holistically assess primate welfare, refine primate care through benchmarking of current programs and identifying areas of improvement, and setting new standards for optimal primate care. The PWAT was developed through internal focus groups with subject matter experts, identifying welfare categories and descriptors based on the primate literature, developing and testing of the tool, and finalizing the tool in a user-friendly platform with automated data analysis and report creation. The tool includes input and outcome-based measures of welfare across 6 categories: physical, behavioral, training, environmental, procedural, and culture of care. The final tool consists of 133 descriptors measured at the room level, site level, and in personnel interviews. The final tool was rolled out to 13 sites for benchmarking in 2022. The overall scores ranged from 63-89.5%, demonstrating that the tool can successfully discriminate between different primate programs and identify strengths and areas for improvements at the individual site level and globally across sites. The results of the assessment are being used to set priorities for primate care improvement and to develop educational materials to address the global challenges with primate management. The assessment will continue to occur biannually to monitor changes over time and quantify the impact of refinements on primate welfare.

Presentation: Oral

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A competency-based approach to teaching animal welfare & the 3Rs to research animal professionals

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Comprehensive animal welfare education is vital to maintaining the highest standards of research animal care. Recognizing that most training was provided during the earliest stages of training, we realized there was a significant need to reinforce and enhance information provided through a high quality, virtual, competency-based certification program in Animal Welfare & the 3Rs. A certificate program was developed based on learner-centered educational theory related to adult workplace education, virtual engagement, and practical application. The program was developed using a competency-based learning framework, identifying the goals of the certificate program, using a gap analysis to identify needed skills, building competencies based on Bloom's Three Domains of Learning to create a well-rounded applicable program that helps learners build competency in cognitive, affective, and psychomotor domains. The program also incorporates backwards design and active learning principles, and learners will be asked to document their competencies in a portfolio. The program is composed of 8 courses: Culture of Care, the 3Rs, Applied Ethics, Animal Welfare, Research Animal Welfare, Animal Welfare Assessment, Humane Interventions, and Compassion Science. The program will be interactive including weekly discussions, reflections and assessments facilitated by a Course Instructor. By providing certification, we are empowering employees to advance animal welfare and 3Rs programs at their own sites and ensuring consistent training on these topics from subject matter experts to maintain high standards of care, even during times of employee turnover.

Presentation: Oral

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Assessing the utility of *in vitro* assessment for acute inhalation toxicity

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Assessing acute inhalation toxicity from formulated products is important for ensuring safety for both workers and consumers. The most common assessments currently are *in vivo* inhalation studies in rodents. To replace animal testing, ensure regulatory acceptance and garner widespread use, alternative assessments must demonstrate equivalent health protection, be relatively easy to implement, and be cost effective.

In an initial study EpiAirway™, a commercially available functional model of the human airway epithelium, correctly identified inhalation hazard categories of three suspension concentrate formulations as having high, moderate, or low inhalation toxicity. Evaluation of irritation potential and tissue damage by transepithelial electrical resistance (TEER), and histopathology assessment were the most sensitive measures.

To further assess the ability of EpiAirway™ to correctly categorize inhalation toxicity, five suspension concentrates and five emulsifiable concentrates were assessed and the protocol optimized. Exposure time was increased to four hours from three, and a 20-hour recovery period added. Evaluations included TEER, LDH release, histopathology and (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) viability.

Under the conditions used EpiAirway™ consistently identified the formulation with respiratory irritation potential as having inhalation toxicity. The results with other formulations required correlation of findings across the assays, across concentrations and where applicable across time. Formulations with moderate to severe eye irritation were over predicted as toxic by inhalation particularly at higher concentrations. Initial results are promising that EpiAirway™ could be optimized and used in a weight of evidence to characterize inhalation toxicity.

Presentation: Oral

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Pharma industry needs for faster adoption of MPS

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Using human-relevant, translational *in vitro* models has been widely considered to reduce attrition during drug discovery and development. Over the past decade a considerable hype emerged regarding the transformative potential of microphysiological systems for pharmaceutical research; yet – while it is agreed that such models could bring value – currently, mostly proof-of-concept studies are available and widespread application is still lacking. Thus, while acknowledging the opportunity and value such human relevant cell systems could provide, the adoption by pharma companies is moderate. Realizing the full potential of these models will need more clear use-cases demonstrating clinical translation, improvements on technical ease of use and greater collaboration between stakeholders. Furthermore, it is proposed that refining existing platforms for specific contexts of use where significant gaps exist in drug development will help broader application, rather than unrealistic claims that microphysiological systems can right away replace the complete drug discovery engine at once. Key advantages of such tissue systems over traditional pre-clinical models, e.g. the ability to mimic human-specific biology such as immunology or defined contexts of rare diseases should be further exploited to establish more use cases that demonstrate true added value. Modeling & analytics can help with back- and forward translation using real world data. Furthermore, the ability to generate patient-derived tissue models will allow personalization of treatments and support precision medicine approaches in clinical trials.

Presentation: Oral

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Establishing scientific confidence of cross-species extrapolations through case studies

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The growing need for non-animal alternatives to toxicity testing has led to the development and evaluation of New Approach Methodologies (NAMs). Key to their acceptance in regulations and risk evaluations, is a demonstration of their relevance and reliability, which are necessary to establish scientific confidence. For over a decade, Interspecies Correlation Estimation (ICE) models, a possible NAMs, have gained scientific acceptance but have yet to be used for regulatory purposes. ICE models leverage existing data and describe mathematical relationships between pairs of species allowing toxicity predictions from surrogates to untested species. Predictions are used to improve assessments by developing Species Sensitivity Distributions from a relatively small sample of empirical data. In this work, we highlight key attributes of ICE models making them suitable NAM candidates and demonstrate their utility through several independent case studies. These case studies included hazard assessments of nonionic surfactants with a shared mode of toxicity, produced water from multiple offshore platforms with global distributions and chemical constituents in biocide formulations used in offshore oil and gas operations. Collectively these and related case studies have consistently demonstrated that ICE models are a promising alternative to toxicity testing, and thus are potentially useful in reducing vertebrate testing and supporting hazard evaluations for chemicals with limited data. The latter has practical implications for regulatory purposes, including registration of existing or new products. In closing, future regulatory acceptance of NAMs could be achieved through the collective outcomes from independent and collaborative case studies involving multiple stakeholders with a shared interest.

Presentation: Oral

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Recommendations for promoting the regulatory acceptance of microphysiological systems

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Microphysiological systems (MPS), as an emerging alternative method to animal testing, have garnered increasing attention for their applications in drug development, toxicity assessment, and disease modeling. A growing body of evidence suggests that MPS has the potential to provide more human physiologically relevant data with lower cost and higher throughput compared to traditional animal models. As MPS continues to advance, regulatory agencies, including the Food and Drug Administration (FDA), are expected to adopt MPS-generated data in the medical product review process, and gradually accept MPS platforms as regulatory and scientific tools. However, the MPS-relevant submission under the FDA framework remains limited in practice. We conducted a comprehensive analysis of the FDA's ongoing activities related to MPS and formulated the following recommendations aiming to facilitate the regulatory acceptance of MPS: 1) establish clear and con-

sistent guidelines, 2) collaborate with academia and industry, 3) provide training and educational opportunities, 4) implement robust validation and qualification protocols.

Presentation: Oral

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Bringing IACUCs into the 21st century: Ethical gaps and room for improvement

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Institutional Animal Care and Use Committees (IACUCs) are responsible for institutional-level oversight of animal research in the United States and similar review mechanisms have been implemented around the world. Part of the goal in establishing IACUC review was to ensure that animal studies meet society's expectations for the ethical use of animals in research. However, almost four decades after IACUCs were implemented in the U.S., significant ethical gaps remain in the process of reviewing animal research activities.

Many IACUCs focus their attention on potential refinements of what the researcher has proposed. Although this narrow scope is permitted by the regulations that govern IACUCs, it is a sharp departure from what the public perceives as the IACUC's role: ensuring that animal studies are ethically and scientifically justified. One of the most striking deficiencies is the lack of any requirement for IACUCs to assess the potential value of the proposed research. Other concerns have also been raised, including the limited representation of non-scientific perspectives, inconsistency of review and lack of concordance between committee determinations, and the lack of transparency around the review process.

Studies regarding public expectations for animal research review will be described and proposals for improving the IACUC review process will be presented. Some of these enhancements have been implemented in other countries with varying degrees of success. Potential challenges will be explored along with recommendations to address them. Cooperation and sustained effort will be necessary to bring IACUC review into the 21st century, but it is well past time.

Presentation: Oral

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Can we justify using nonhuman primates in neuroscience research? A costs versus benefits discussion

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The justifications for using nonhuman primates in biomedical research often begin by indicating only minimally harmful, maximally impactful, scientifically necessary research are being conducted. Unfortunately, in the case of neuroscience research, which accounts for approximately 50% of all non-regulatory experimentation with primates, these criteria are rarely met.

Neuroscience experimentation inherently involves inflicts irreversible harms on the animals. Surgical insertions of intracranial electrodes, drug delivery or neuron-manipulating apparatus, and skull-affixed head-stabilizing equipment are standard procedures in primate laboratories. Behavioral assays often involve the use of food and water deprivation and prolonged physical restraint, and animals frequently have their nervous system permanently damaged to induce symptoms of human neurological conditions. Primates in these labs are also subject to maternal deprivation, impoverished environment, and decades of complete social isolation.

The physiological and psychological effects of holding emotionally and behaviorally complex primates captive in a laboratory environment disrupts both the neural systems and the behaviors being assessed. Coupled with artificially induced symptoms, macroscopic and microscopic differences in the brains of different primates, human-specific developmental mechanisms and trajectories, and critical differences in gene expression across species, the likelihood of data from captive nonhuman primates translating to humans is limited and difficult to predict in advance.

Here we will review the current landscape of nonhuman primate use in neuroscience, including the numbers of animals used, common procedures, the scientific limitations of these studies and several viable non-animal alternatives, and also weigh the harms versus the benefits of conducting non-clinical, non-regulatory experiments on our closest relatives.

Presentation: Oral

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Pain management via the drinking water: A watertight affair?

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Purpose: Due to its non-invasive nature, voluntary oral self-administration via drinking water might represent a favorable administration route for analgesic drugs in rodents. However, factors like limited acceptance and inconstant fluid intake might hamper the achievement of sufficient drug plasma levels. Aim of this study was to assess oral acceptance, plasma concentration-time curves and tolerability of standard analgesics.

Experimental approach: Carprofen, metamizole, buprenorphine, or tramadol were administered via drinking water to female and male rats (overall n = 159) and mice, with mice additionally treated with meloxicam and butorphanol (overall n = 252). Analgesic target dosages represented the maximum recommended dose according to the current expert information on pain management for laboratory animals of the German Society of Laboratory Animal Science.

Summative findings: Drug-containing solutions were generally well accepted by mice, resulting in stable analgesic plasma levels in most cases within 12-24 h after treatment start. Only meloxicam required sweetening, showed distinct circadian influence on plasma levels and was associated with moderate side effects. Rats showed dislike of tramadol and almost completely refused metamizole intake. While carprofen was well tolerated, buprenorphine and tramadol treatment were accompanied by distinct behavioral side effects including pica behavior.

Conclusions: Administration of analgesics via drinking water requires close monitoring to ensure constant uptake of the target dose. Certain analgesics cannot be recommended in the explored doses due to adverse effects or limited intake. Overall, rats appear to be more susceptible to side effects and taste changes than mice.

Presentation: Oral

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Novel *in vitro* tri-culture hepatic model to evaluate human relevance of chemical-induced thyroid toxicity

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Increased regulatory requirements to assess human safety for pesticides, combined with the need of suitable alternative approaches that replace, reduce and refine animal models, outlined the development of an *in vitro* model to evaluate the human relevance of chemical-induced liver and thyroid effects. The ability to distinguish chemicals that cause direct toxicity from those that cause indirect effects through liver enzyme induction mediated by nuclear receptor (NR) activation in rodents is key. This study focused on evaluating thyroid hormone (TH) metabolism effects after exposure to six known liver enzyme inducers: Phenobarbital (PB), Rifampicin (RIF), Pregnenolone 16α-carbonitrile (PCN), Polychlorinated biphenyl 153 (PCB153), CITCO and Dexamethasone (Dex) at non-toxic concentrations. An *in vitro* tri-culture model, comprised of primary hepatocytes (human and rat), endothelial and stromal cells, was used. Physiological functionality was assessed using urea and albumin. CYP and UGT gene expression was evaluated after exposure to the reference compounds by RT-qPCR. T4 clearance and its metabolite concentrations (T4G, T4S, T3) were measured by LC-MS/MS. Results demonstrated stable urea and albumin concentrations over 10 days. Consistent CYP and UGT gene expression of known NR agonists was shown for both human and rat. T4G was increased in both species, but higher in rat, while T4S was undetectable in both species and T3 was only increased after RIF exposure in human. T4 clearance increases after Dex, PCN and PCB153 exposure in rat, and after RIF exposure in human, demonstrated species-differences. The model shows promise, and expanded testing with additional donors and further optimization are planned.

Presentation: Oral

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Macaques over the edge

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A 2013 report issued by the U.S. National Institutes of Health confirmed that experimentation on chimpanzees is unnecessary and that "research involving chimpanzees has rarely accelerated new discoveries or the advancement of human health for infectious diseases." Given these findings it seems highly suspect that other primates, with whom we share less evolutionary history, would offer more reliable data. Yet the primate biomedical industry persists in its commitment to importing tens of thousands of long-tailed macaques each year. Neither the origin of the long-tailed macaques supplied to meet this demand, the risks posed to the people capturing and handling the monkeys, the effects of the monkeys' removal on the ecosystems from which they were taken, nor the consequences of using poorly characterized, wild-caught macaques are considered. In 2022, the global conservation status of long-tailed macaques, the most intensely "harvested" primate for use in biomedical research, was raised to endangered following assessment by the International Union for Conservation of Nature's Red List of Threatened Species. A five-year investigation by U.S. authorities has also alleged illegal activities by entities involved in the global trade of macaques for primate experimentation. Macaques are seed dispersers making them a keystone species in the environment; remove the monkeys from the forest and you risk a cascade of ecological consequences. The role that the global trade of live primates/primate tissues for use in biomedical research could play in the emergence of pandemic threats has, until now, managed to escape scrutiny.

Presentation: Oral

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Private funding and support for early career researchers in non-animal methods: Strategies for success

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Early Career Researchers (ECRs) in any scientific area face challenges; however, ECRs interested in non-animal research methods can face additional obstacles when launching their research careers. In many fields of biomedical science and toxicology, animal research methods remain the default approach and ECRs who focus on non-animal methods sometimes find fewer funding opportunities to support their work and reviewers who expect a higher degree of evidence for alternative approaches.

Private funding organizations dedicated to the development of NAMs can help address this gap and support ECRs in creating a solid foundation for their research programs. Private funders can tailor their review criteria to prioritize the potential impact in reducing or replacing animals, which might not be a high-priority area for other funders. Furthermore, these programs provide funding mechanisms where proposals for non-animal approaches do not directly compete against more traditional animal research studies for the same funds.

This presentation describes the role private funders can play in supporting the development of NAMs and the careers of ECRs. It will survey available funding opportunities, share advice for preparing a competitive proposal, and provide strategies to help ECRs leverage this type funding to support their long-term career plans. Private funding targeted at non-animal approaches can provide crucial support for obtaining preliminary data or proof-of-concept, which can serve as a springboard for sustainable, longer-term funding. Helping ECRs understand and successfully compete for funding can play a key role in setting them up for success.

Presentation: Oral

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Education and training in laboratory animal science in the European Union: Benefits, challenges, and solutions for mutual recognition

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Working with animals in scientific procedures requires adequate education, training, competence, and the maintenance of competence in laboratory animal science. This is essential for advancing scientific research while ensuring the welfare of laboratory animals. The EU framework on education and training strives for more aligned standards to facilitate mutual recognition and the free movement of personnel and scientific exchange. This presentation will provide an overview of common situations related to mutual recognition, including the benefits and challenges of harmonization. The talk will also address potential barriers to mutual recognition and propose some solutions to overcome common obstacles.

Presentation: Oral

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The European Partnership for Alternative Approaches to Animal Testing (EPAA): Accelerating the transition to animal-free, sustainable innovation

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The European Partnership for Alternative Approaches to Animal Testing (EPAA) was established in 2005 as a unique collaboration between the European Commission and industry stakeholders that brings together 5 DGs of the European Commission, 38 companies and industry associations from 7 sectors (agrochemicals, animal health, chemicals, cosmetics, fragrances, pharmaceuticals, soaps & detergents) and 3 European Agencies (ECHA, EFSA and EMA).

EPAA seeks to accelerate the transition to animal-free, sustainable innovation through promoting the development and acceptance of 3R methods; fostering cross-sector knowledge-sharing; increasing international collaboration and facilitating stakeholder dialogue. EPAA strategic priorities are set by the Steering Committee (representatives from the European Commission services, companies, and industry associations) taking into account input from the Mirror Group (academics and civil society representatives).

EPAA has 7 projects evaluating and promoting alternative approaches covering: New Approach Methodologies (NAMs) in regulatory decisions for chemical safety; Skin Sensitisation NAM User Forum; Carcinogenicity of Agrochemicals; Acute Toxicity; Harmonisation of 3Rs in Biologicals; Monoclonal Antibody Safety Testing; and Human Rabies Vaccine testing. In 2023, EPAA will also: support the development and implementation of an EU roadmap to replace use of Animals for Regulatory Testing of Chemicals; organise a cross-sector Partners Forum to review NAM use for Environmental Safety Assessment; and organise an exhibition at the EU Parliament to share progress with MEPs. This presentation will report progress and share learnings from EPAA's ongoing activities.

Presentation: Oral

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Making NAMs the ethical solution to animal use in the pharmaceutical industry

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The phasing-in of New Approach Methodologies (NAMs) in the pharmaceutical industry is motivated by the need for more predictive models to accelerate the discovery, safety and efficacy assessment of drug candidates ahead of clinical trials. NAMs are human-derived *in vitro* or *in silico* models (e.g., organoids, gene editing and artificial intelligence) that have the potential to reduce or replace animal models as primary proxies for human biology.

Ethical and regulatory frameworks supporting the use of animals in biomedical science follow a utilitarian approach whereby any inflicted harm is expected to be outweighed by health benefits for the wider population. Despite having played an instrumental role in life sciences, the translatability of *in vivo* studies has been repeatedly challenged on scientific grounds, therefore questioning the morality of animal use. While NAMs may alleviate ethical concerns pertained to animal use, they also raise significant and new ethical challenges for which there is no established bioethical framework.

This work aims to present the ethical challenges inherent to NAMs and outline four principles laying the foundations of an ethical framework. The four principles are i) scientific quality, especially translatability to patient populations ii) acceptability from the patients, the public, and regulatory bodies, iii) equity of drug accessibility and iv) environmental sustainability. The proposed framework would provide proactive bioethical guidance evolving as the NAMs develop, taking stakeholders' needs into account and decreasing the reliance of the pharmaceutical industry on animal use for innovative drug discovery.

Presentation: Oral

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ETPLAS - FELASA join forces to establish a CPD framework for the EU

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A working group under the EU funded European Training Platform for Laboratory Animal Science (ETPLAS) Preparatory Action Grant Project is tasked to draft guidelines for the harmonisation of Continuing Professional Development (CPD) in LAS for researchers across the EU to contribute to free movement of researchers.

For these guidelines we will focus on the role of the course organisers and also the role of the competent authorities. A harmonisation of CPDs should involve both groups of people.

A FELASA task force aims to revise their 2010 guidelines for CPD of scientists involved in animal experiments. These two projects are complementary as they cover all groups of people involved in CPD. Therefore, they joined forces.

The first step was to define what can be considered CPD as the basis for these guidelines. This was deemed necessary since, within the EU, education and training have been devolved to the individual member states (MSs). Consequently, EU MSs have different E&T systems in place including those for CPD. The aim and challenge will be to propose a framework that will be acceptable to all. The framework will also cover a proposal for training tools for course organisers, a common CPD certificate to facilitate the mutual recognition of CPD activities. The results of an online survey and a first version of the framework will be presented.

Presentation: Oral

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A platform for identification of chemicals for which fish have high sensitivity

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Predicting if fish species are more or less sensitive to a given chemical than other aquatic taxa could lead to a significant reduction of fish *in vivo* toxicity testing requirements for the assessment of chemical safety. Our research aimed at establishing taxon-specific sensitivity to different chemical modes of action by comparing existing chronic toxicity data generated for fish, invertebrates and algae. To facilitate this inter-species comparison, we developed a novel informatics knowledge platform that allows the rapid integration, analysis, and visualisation of large multi-type datasets, enabling evidence synthesis. The platform currently includes multi-dimensional data for 1,493 chemicals of which 1,170 are active pharmaceutical ingredients (APIs), and comprises 1) ecotoxicity data, 2) physicochemical, mechanistic, and pharmacological data, and 3) drug-target, target-pathway, target-phenotype, target-disease associations. Network analysis of drug-toxicity and drug-target interaction data was used to identify molecular targets associated with high fish-specific toxicological sensitivity. The EcoDrug tool was used to evaluate the evolutionary structural and functional conservation of drug targets. To interpret the functional role of priority targets, multiple databases were used to retrieve target-pathway and target-phenotype associations across species. This data-driven evidence synthesis approach was used to assign a confidence judgment to each association and ultimately identify a set of priority molecular initiating events (MIEs), Key Events (KEs), and Adverse Outcomes (AOs). Fish displayed higher toxicological sensitivity than other species for 53% of APIs. The analysis of the poly-pharmacology network revealed that 68 drug targets are consistently associated with high fish sensitivity compared to other taxa.

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Presentation: Oral

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Establishing the credibility of computational models in biology and toxicology

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Computational models are often constructed to aid people's understanding of biological processes or to inform decisions related to safety and efficacy of new therapies. The growing sophistication and applicability of computational modelling have created a strong demand for their widespread use by industry for product development and for demonstrating regulatory compliance. However, this presents a particular challenge, since developers and end-users rarely share the same level of confidence in a computational model. Although model verification and validation can help bridge the gap, the ultimate aim is to achieve a sufficient level of scientific credibility which reflects the willingness of people to trust a model's predictions and make decisions based on them. Establishing the credibility of computational models typically boils down to understanding their testability and epistemic foundation, in order to guide a validation process to address evidence gaps. Credibility factors, grounded in the philosophy of science and relatable to different validation frameworks in use today, are a useful way of organising the evidence base underpinning credibility, and understanding how different weights can be applied to different factors, depending on the nature of the computational model and the needs and perceptions of the end-user community. Another essential element of establishing credibility is addressing social epistemology, that is, designing a process that aims to maximise the degree to which developers and end-users share a common understanding of the model and the evidence presented.

Presentation: Oral

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Training the next generation of researchers in the organ-on-chip field

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Organ-on-Chip (OoC) systems are by definition "fit-for-purpose microfluidic devices, containing living engineered organ substructures in a controlled microenvironment, that recapitulate one or more aspects of the organ's dynamics, functionality and (patho) physiological response *in vivo* under real-time monitoring". They promise great potential for human-relevant science, personalized medicine, pharmaceutical R&D and can be a game-changer towards the 3Rs, hence the transition to non-animal science.

The OoC field is characterized by a high degree of interdisciplinarity and draws upon technologies and concepts from fields such as engineering, biology, physics and clinical science. However, this relatively new technology still deals with several challenges currently impeding the broad adoption of OoC in academic, clinical and industrial research. To overcome them, it is imperative to train experts at different levels of education and work function.

By using a questionnaire, we aimed to identify the key aspects for training such as stakeholders to be trained, specific skill sets and the adequate types of training required to prepare future experts to advance the OoC field and change the way how research is performed. The information collected and analyzed in our study serves to guide training initiatives for preparing competent and transdisciplinary researchers, capable of assuring the successful development and application of OoC technologies in academia, industry, personalized medicine and clinical trials on chip.

Training the next generation of scientists, technicians and policymakers is challenging and requires a tailored effort but will pay off in well-qualified and competent transdisciplinary professionals who will continue exploring and successfully applying OoC technology.

Presentation: Oral

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The rise of European 3R Centres and their network EU3Rnet

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EU3Rnet is an open network for European 3Rs centres and platforms. The 3Rs centres and platforms are very important points of contact and play an immense role in their respective countries as "on the ground" facilitators of Directive 2010/63/EU. They are also invaluable for the widespread dissemination of information and for promoting implementation of the 3Rs in general [1]. In this contribution, the development of the EU3Rnet is presented and the history of the European 3Rs centres is recapitulated. A further focus is set on special features, but also commonalities of the 3Rs centres and platforms, especially with regard to organization, structure and topics. The first EU3Rnet consensus statement and the publication "The rise of Three Rs centres and platforms in Europe" are presented as previous highlights [1,2]. Recently, the COST Action IMPROVE with the topic "3Rs concepts to improve the quality biomedical science" was approved, which will strongly support the networking in the 3Rs area. This activity will be presented in detail with its current status and activities, plans, possibilities and instruments. The working groups will focus on Quality and Translatability of Science, Implementation, Dissemination and Education, whereby ethics is an integral part in the work of the single working groups. Cooperation and active involvement in the COST Action will be invited within the framework of this open bottom-up network approach.

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Presentation: Oral

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Towards NGRA: Skin sensitization blazes a trail

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Regulatory and ethical demands have driven the need for developing a Skin Sensitization Next Generation Risk Assessment (NGRA) based on New Approach Methodologies (NAM) and Defined Approaches (DA). Much scientific build-up work was done by the Skin Tolerance taskforce under the Cosmetics Europe LRSS program and continues under the International Collaboration on Cosmetic Safety (ICCS). Here, we briefly recap the journey towards the current updated framework and its application to several case studies with increasing complexity.

Based on a systematic evaluation, the most promising NAM (*in chemico* / *in vitro*) were selected and assessed in detail by generating and compiling a NAM database, which is continuously being expanded. It provides the basis for evaluations of NAM and DA, and it builds the backbone for NGRA case studies. A series of case studies of increasing complexity, and stepwise incorporating learnings, has been conducted. The most recent case study example focused on use of inconsistent NAM information leading to differences in the hazard and potency information obtained from the respective DA and affected the confidence in the risk assessment decision for different consumer use scenarios.

Going forward, case studies focusing on read-across, toxicokinetics and uncertainty assessment will further strengthen the NGRA. This journey of developing a fit-for-purpose NGRA framework for skin sensitisation has come a long way, bringing about many valuable lessons.

Presentation: Oral

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Building trust and increasing utility of NAMs through preregistration and open protocols

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In recent years, non-animal models (NAMs) developed into highly complex and disease relevant alternatives to animal models. For these advanced NAMs to effectively replace animal experiments, they need to be adopted at scale. For this, potential adopters need to trust results from published studies and be able to implement the models in their lab. In other biomedical fields like clinical trials, two strategies have been implemented that ensure trustworthiness and transparency of published results: 1. preregistration of study design and analysis and 2. publishing of study protocols. These strategies need to be meaningfully adapted to NAMs. I will present challenges and case studies in establishing such processes from a 3R Center in Berlin. Specifically, I will illustrate the utility of protocol peer review and publishing within the consortium. I will further address challenges and some solutions for establishing preregistration in a research field where fast-paced experimental series consist mainly of trial and error learning.

Presentation: Oral

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Development of a scale to classify the animal-free status of *in vitro* tests: A tool for transparent communication

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In vitro methods commonly use animal derivatives including serum, antibodies and tissue extracts, raising the question, "What is truly animal-free testing?" As well as ethical concerns, these additives can compromise reproducibility of tests and relevance to human physiology. Here, we provide guidance on truly animal-free *in vitro* testing and describe some of the principles followed in our laboratory. We introduce our classification scale, which allows the animal-free status of methods to be clearly identified. We define 7 categories of tests, which increasingly eradicate animals and animal components to achieve fully defined conditions. Level 1: *in vivo* live animal testing. Level 2: *in vitro* with components that involved live animal suffering (e.g., FBS). Level 3: *in vitro* with components that required "humane" animal sacrifice (e.g., rat liver extract). Level 4: *in vitro* with components that are waste products of the meat industry (e.g., gelatin, bovine cornea). Level 5: *in vitro* with components that have previously been exposed to animal product (e.g., human cell lines previously cultured in FBS; some human tissue models). Level 6: *in vitro* animal-product-free with components derived ethically from humans. Level 7: *in vitro* animal-product-free, fully defined. The scale can provide useful context for goal-setting in moving towards animal-product-free and defined *in vitro* systems for enhance human relevance. It is also a valuable tool for stakeholders to communicate accurately and transparently about the ethical status of a test in terms of animal welfare – an important factor in the global trend towards vegan products.

Presentation: Oral

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Update on Canada's efforts to reduce the reliance on animal toxicity testing

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Canada has taken concrete actions to reduce reliance on animals in toxicity testing and research, through renewed legislation, policy innovation, harmonization with international activities and advancing research on New Approach Methods. On the legislative and regulatory front, amendments have been introduced, through Bill S-5, to the Canadian Environmental Protection Act to reduce reliance on vertebrate animal testing when conducting research and assessing the risks that substances may pose to human health and the environment. Furthermore, the Government of Canada is working towards banning the testing of cosmetics on animals. Government of Canada scientists actively participate in global efforts to develop, validate and implement effective non-animal methods to ensure reliable, harmonized methods are available for regulatory information needs, including hazard/risk assessments. Additionally, Canada regularly provides expertise to support peer review and validation of new non-animal methods, and for case studies supporting international (OECD, EU, US) test guidelines and guidance development. Together with the Canadian Centre for Alternatives to Animal Methods at the University of Windsor,

whose work includes the development, validation, and promotion of alternative methods for biomedical research, academic instruction and regulatory testing, this presentation will provide an update on Canada's continued efforts to explore alternatives and next steps to further reduce reliance on animal testing.

Presentation: Oral

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Characterizing the cytotoxic and molecular effects of environmentally relevant pesticides on human Caco-2 and HepG2 cell lines

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The widespread use of pesticides is of concern to ecosystem and human health. Animal-based testing of chemicals like pesticides is challenged they are resource-intensive, time-consuming, and unethical. These concerns have called for the need to explore New Approach Methods (NAMs) to replace, refine, and reduce animal research. The objectives of this study were to: (A) measure the cytotoxicity (i.e., LC50) of 19 pesticides considered important to Canadian ecosystems on the human Caco-2 (intestinal) and HepG2 (liver) cell lines; B) characterize molecular responses in both cell lines to derive transcriptomic points of departure (tPODs); and C) compare the resulting tPOD values with the *in vitro* LC50 values and data from the literature to determine if the tPOD values are protective of adverse outcomes. Cytotoxicity results based on Alamar Blue tests found that chlorothalonil and imazethapyr were the most toxic pesticides studied for Caco-2 cells, with the LC50 values below 20 µM. HepG2 cells were comparatively less sensitive to the tested pesticides, where the LC50 values for the most toxic pesticides, chlorothalonil and diquat, were above 100 µM. Changes in gene expressions will be measured by ultraplex RNA sequencing technology, from which tPODs will be calculated. This research is expected to help support the method development of *in vitro* studies as alternatives to animal testing strategies.

Presentation: Oral

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An outside the box approach for housing rats to enhance their behavioural repertoires

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There is increasing emphasis on the importance of natural behaviours and postures on the health and wellbeing of research rats. We investigated behavioural and physiologic differences between rats housed in different environments and the interaction between housing and habituation to handling. SD rats (n = 70, 34M, 36F) were randomly assigned to housing treatments: standard cage (C): n = 3 rats/cage, 8 cages or primate cages modified for housing rats (T): n = 5-6 rats/cage, 8 cages. All rats received 15 s of gentle handling 3 days/wk over the 18-day study. At study end, rats were assessed for anxiety (elevated plus maze (EPM)) and novel human approach test before/after restraint for blood collection. Blood glucose levels assessed response to restraint and body weight was also monitored. Duration of general behaviour and posture were scored daily from video recordings. Data were analyzed using linear mixed models. Treatment, sex, and time were included as fixed effects and cage was the random effect. There were no weight differences between C and T rats ($P > 0.05$), no differences in blood glucose levels in response to restraint ($P > 0.05$) and no difference in latency to touch novel human before/after blood collection ($P > 0.05$). T rats visited the open arms of the EPM more frequently ($P = 0.039$), suggesting less anxiety-like behaviour. T rats were less inactive ($P < 0.0001$), spending more time moving ($P < 0.0001$) and exploring resources ($P = 0.0003$). T rats also spent less time lying ($P < 0.0001$) and sitting ($P = 0.0006$). These results suggest that more complex housing is beneficial to rats, allowing more active behaviours and postures than standard housing.

Presentation: Oral

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Development and execution of an occupational next generation risk assessment (NGRA) on an exclusive use cosmetic ingredient under EU REACH: A case study on C12-15 alkyl benzoate

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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 some examples in the literature highlighting NGRA for consumer safety assessment of cosmetic ingredients (Dent et al., 2018; Baltazar et al., 2020), but none currently outlining how this might be done to assure occupational safety. Therefore, in this case study, NGRA was applied in an occupational safety assessment for an ingredient exclusively used in cosmetics (INCI: C12-15 Alkyl Benzoate), in order to avoid animal testing requested under the EU REACH regulation, following a compliance check from the European Chemicals Agency. Modelling was used to estimate worker external dermal and inhalation exposure to the substance from handling, during formulation into finished cosmetic products. These external exposure estimates were combined with *in vitro* ADME data and converted to internal concentrations (plasma Cmax) using physiologically based kinetic (PBPK) modelling. Systemic toxicity was assessed using a suite of *in vitro* NAMs to identify points of departure (PoDs) for a variety of biological effects and bioactivity. These assays indicated C12-15 Alkyl Benzoate exhibits little bioactivity, and enabled bioactivity:exposure ratios (BERs) to be calculated which prove it is safe for workers and that risks are adequately controlled under normal occupational use conditions. This case study highlights how an NGRA approach can be used to reach an occupational safety decision and formulate a scientific basis to avoid animal testing under EU REACH and similar schemes.

Presentation: Oral

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A next-generation framework for agrochemical risk assessment

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Historically, agrochemical toxicity testing has been rooted in strict regulatory requirements structured by legislation – most of which were enacted before the sequencing of the human genome. Over time, several regulatory authorities have proceeded with legislative reform, including an ongoing commitment towards the 3Rs: replace, reduce, and refine animal studies to the extent possible. This has catalyzed efforts for identifying collaborative opportunities to fulfill legal data requirements by leveraging 21st century approaches to safety assessment. Today, the safety evaluation community finds themselves in a paradox where new tools and approaches are available to be used in an integrated approach to evaluate potential toxicity; however, these tools, unlike the currently established approaches, must be proven to be equally, if not more, protective of public health than standard animal test guidelines and demonstrated to be fit for regulatory purposes. To help build scientific and regulatory confidence as well as public trust in new approach methodologies (NAMs) and technologies used in the plant protection product space, the Committee on Transforming the Evaluation of Agrochemicals (a multistakeholder project of the Health and Environmental Sciences Institute) is developing a framework towards a fit-for-purpose, next generation safety assessment paradigm. The framework is being developed with the underlying objective of supporting the uptake of NAMs that demonstrably fulfill regulatory data needs. This presentation will provide an overview of the framework, the overall approach for this multi-year initiative, and describe the mindset that is now required to guide another reform in the safety evaluation of agrochemicals.

Presentation: Oral

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What policy and science tools are needed to effectively transition away from animal models

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Around the world, the 3Rs are more enshrined than ever in animal welfare legislations; regulations; research policy etc., yet the numbers of animals used in research is remaining level or on the rise in many countries. The increase in legislative protections signals

a greater public desire and government willingness to transition to human-centered research and testing methods. However, this desire does not appear to have produced a marked reduction in animal use in biomedical research. This presentation will examine the multiple and interconnected causes of various nature (including scientific, social etc.) of this "wicked problem". It will be argued that obtaining more effective results towards a faster transition towards non-animal models in biomedical research will require a holistic "systems thinking", providing a better understanding of how different actors and institutions are interacting with each other. Examples of policy and scientific initiatives will be offered to demonstrate how system thinking can inform strategies to increase investment and use of human-centered research methods while gradually moving away from animal models.

Presentation: Oral

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20 years of learnings from a corporate animal welfare and reporting program

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Shell has been an industry leader in animal welfare reporting for the past two decades. We were the first company to track and keep record of all vertebrate animals (e.g., rats, mice, rabbits, birds, fish, frogs) used for product safety, regulatory, and compliance purposes and reporting these numbers externally with complete transparency. During this time, Shell has been strongly committed to end the need to do testing involving animals and has prioritised research into alternative methods. Shell strives to replace animal testing with suitable alternatives, while ensuring that we continue to innovate, develop, and maintain safe new products and technologies. The aim of this review is to present an overview of how the program has matured against the landscape of animal testing requirements and the constantly changing regulatory and business environment and pressures. We will also share a comprehensive summary of our annual animal use data collected from the past two decades and how these numbers have helped shape our strategy towards the goal of one day reporting zero use of animals.

Presentation: Oral

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Preliminary metabolomic and toxicological studies of environmental contaminants – Organophosphates (OPs), brominated flame retardants (BFRs) and poly-fluoroalkyl substances (PFASs) – Detectable in waste of electrical and electronic equipment (WEEE) plants – The VAISAL project

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Waste of electrical and electronic equipment (WEEE) is an emerging threat for environmental and human health due to the occurrence of toxic organic and inorganic chemicals in e-waste components. The VAISAL project (INAIL BRiC2019 - ID13 grant) aims to identify such chemicals and investigate toxicological and metabolic responses, also considering synergistic effects.

Hence, particle matter (PM) samples were collected in three Italian WEEE plants by 14-stage cascade impactors: inorganic and organic compounds (23 and 101 analytes, respectively) were characterized. Untargeted analysis workflows were applied to the same PM extracts using liquid-chromatography coupled to high-resolution mass spectrometry to detect further organic contaminants. Finally, some targeted and non-targeted organic compounds have been subjected to cell-based bioassays in two human-derived cell lines, A549 (epithelial bronchial cells) and HepG2 (hepatocytes), in order to perform in a dose-dependent manner (1pM-100microM range): i) cytotoxicity by MTS assay, ii) preliminary untargeted lipidomics analysis. In addition, metabolic degradation by human liver microsomes, human skin microsomes and human liver hepatocytes were also performed.

Obtained data will be presented focusing on the role of some organophosphates (OPEs), brominated flame retardants (BFRs) and per- and poly-fluoroalkyl substances (PFASs). In particular, in the *in vitro* models (A549 and HepG2), representatives of two PM

routes of exposure, only PFASs shown some cytotoxicity at concentrations ≥ 1microM, whereas tested BFRs and OPEs did not show any cytotoxicity. In addition, second generation PFASs were characterized by a high metabolic stability, while for OPEs the full metabolite identification led to identify most common metabolic degradation pathways.

Presentation: Oral

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Applying the 3Rs to fish acute toxicity tests in an industrial chemical company

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Acute toxicity tests in fish are currently conducted as a part of regulatory risk assessment packages for the assessment of ecological safety of chemical products and they are also used in Canada and the United States to assess for effluent toxicity of discharges. In reviewing its vertebrate use Shell identified that use of fish in tests for either product testing or whole effluent toxicity testing, was by far the largest category of vertebrate animal testing conducted by Shell, accounting for 87% of the total vertebrates used in 2021. From this information Shell developed its own 3R's inspired strategy to address the 3Rs of reducing, refining, and replacing fish testing. This approach includes: 1) Review Numbers – understanding where fish are used to identify where research, refinements, and advocacy efforts should be targeted; 2) Research & Development – conduct research on alternative methods and approaches that can be used to replace standard methods; 3) Reaching out – work with stakeholders and peers in targeted scientific, regulatory, and industry forums to foster the adoption of alternative approaches for fish. An overview of Shell's strategy to achieve the 3R's for fish testing will be presented along with examples and experiences with some of these alternative methods, including a combination of *in silico*, *in vitro* and *in vivo* techniques. It may be that no single method or test by itself is enough to replace fish testing but using a weight-of-evidence approach could help to refine and reduce fish testing considerably.

Presentation: Oral

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3D placental trophoblast fusion and invasion model for drug toxicity studies

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During normal pregnancy, placental trophoblast cells differentiate into two major lineages: syncytiotrophoblasts (STs), responsible for placental barrier function, and extravillous cytotrophoblasts (EVTs), responsible for uteroplacental vascular remodeling. Failure or impartial maturation of these cells is often reported in cases of pregnancy complications, preterm labor and fetal death. Drugs and other exogenous substances have been shown to disrupt the differentiation and function of these cells, driving the need for predictive *in vitro* placental models. In this work, we sought to construct a 3D perfusable model of placental chorionic villi to assess

the effects of commonly prescribed drugs on trophoblast cell function and differentiation. To do this, we designed a placental channel with villus-inspired structures embedded into the fibrin hydrogel of our lab's proprietary AngioPlate device. This channel was seeded with blastocyst-derived placental stem cells which were then differentiated into ST and EVT lineages to produce trophoblast-lined perfusable channels. We then characterized the cells' capacity to differentiate into both syncytiotrophoblast and extravillous cytotrophoblast cells, by quantifying fusion efficiency and degree of invasion (immunofluorescence). Once our healthy model was established, we challenged it with physiological concentrations of commonly prescribed drugs. We observed significant changes in ST barrier integrity (dextran permeability assay) and EVT invasion capacity (cell tracking), demonstrating the potential utility of this model in drug safety studies. Further, our placental AngioPlate model is compatible with standard 384-well plate cultures and can be scaled up to meet the needs of high-throughput studies.

Presentation: Oral

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Validating the wildling mouse model: An interdisciplinary approach combining experimental and meta-research methods

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The Wildling model is a new mouse model with a natural microbiome that can improve the generalizability and translational potential of preclinical models by better reflecting human immune system interactions.

To systematically evaluate the potential of this model, Charité 3R funded a consortium bringing experimenters and meta-researcher together. The project aims to prepare an evidence-based decision about the extent of Wildling facilities at Charité. To do so, the potential of Wildlings will be assessed through an experimental evaluation of the external model validity in various disease models including influenza, lung infection, kidney injury, cystic fibrosis, Alzheimer, cancer, and stroke. Uniquely, the consortium is complemented by a meta-research and support project that ensures robust evidence generation, high scientific rigor, and harmonization of procedures and score sheets. We will investigate, how disease characteristics are recapitulating in the Wildling model compared to (established) e.g., specific pathogen-free models and most importantly to humans. In addition, we will assess the efficacy of a given treatment considering (i) a different disease onset and (ii) the immune response to the treatment.

The interdisciplinary work environment will allow us to not only gain knowledge on the potential of the Wildling model(s) in a disease-dependent manner but also to generate evidence about their validity to allow an informed decision about future capacities and research on Wildlings. Overall, this study will provide valuable insights for other medical centers interested in incorporating and validating a new disease model like the Wildlings into their preclinical research.

Presentation: Oral

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Development of non-animal methods for potency testing of diphtheria and tetanus vaccines

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Animal potency assays for many vaccines suffer from inherent variability resulting in relatively poor discriminative power, which makes them unsuitable for routine batch control. However, in the absence of validated alternative assays, large numbers of animals are still required globally for the routine control of these vaccines. As part of the VAC2VAC project, we have developed ELISAs that are intended to measure antigen content and quality in diphtheria and tetanus vaccines. The assays use well-characterized monoclonal antibodies that are directed against relevant epitopes on the target antigen. Proof of concept for use as an *in vitro* potency test has been demonstrated using representative vaccine products from different manufacturers and we have shown that the assays are able to detect quality defects in vaccines that were deliberately introduced (for example through exposure to elevated temperatures). The ELISA for tetanus vaccines has been shown to be applicable to a wide range of veterinary vaccines, thus increasing the scope for use of the alternative assay for tetanus vaccines across both the human and veterinary vaccine industry. The assays are precise and reproducible and have been successfully transferred to other laboratories as part of ongoing efforts to facilitate validation and regulatory acceptance. Once validated for a particular product, these assays have potential to replace the current animal potency test(s) for the purposes of routine batch control and could significantly reduce animal usage going forward.

Presentation: Oral

716

Application of refinement to reduce the number of primates used in biomedical research and improve their welfare

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When there are no alternatives to using primates in regulated research and testing, they are subject to scientific procedures that have the potential to cause harm. These harms, combined with keeping primates in laboratory conditions, are balanced against the potential to improve the quality of life of animals and humans gained from their use. There is a growing body of evidence demonstrating the inextricable link between animals' welfare with experimental outcomes affecting the accuracy and reliability of scientific output. Refinement can be used to mitigate harms across their lifetime by improving breeding conditions, trial design, environment, sociality, as well as introducing opportunities for choice through play or cooperative training. Taken together, these components form a comprehensive program in primate behavioral management that can be applied across disciplines with the advantage of promoting a strong culture of care. Alongside the direct welfare benefits, there is empirical evidence in human clinical medicine that patient-centric care results in better diagnostic and clinical outcomes, reduced anxiety and distress, adherence to treatment, and stronger patient satisfaction. Evidently these same factors can affect the validity of an animal model, so it is logical to equally prioritize in the non-clinical setting to avoid costly translational failure of promising therapies. Refinements must be integrated at all stages of a primate's life to improve their welfare by providing opportunities for positive experiences and conditions that enable effective coping. We present evidence-based welfare and scientific benefits resulting from the successful application of Refinement and subsequent impact on achievement of Reduction.

Presentation: Oral

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Effects of *Lactobacillus plantarum* on a human induced pluripotent stem cell derived intestinal epithelial cell model in comparison with human *in vivo* data and a Caco-2 cell based model

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Some commensal bacteria, like *Lactobacillus plantarum*, stimulate the immune system and could play a role in the induction of immune tolerance. The human colonic adenocarcinoma cell line Caco-2 is a widely applied model to study effects on the intestinal barrier. This model comprises enterocytes and lacks a mucus layer and may not adequately represent human physiology. Human induced pluripotent stem cell (hiPSC)-derived intestinal epithelial cell (IEC) layers contain a mucus layer and consist of different intestinal cell types. They may represent a more physiologically-relevant model to study effects of bacteria, but so far limited data is available comparing the IEC model, the Caco-2 model and human *in vivo* data.

Immune-related responses were compared between the IEC and Caco-2 model. Exposure to an inflammatory cytokine cocktail increased expression of antimicrobial peptides and pro-inflammatory cytokines to the highest extent in the IEC model. To evaluate whether *L. plantarum* affects immune responses, both models were exposed for 24 h to *L. plantarum* before adding the inflammatory cytokine cocktail. Compared to inflamed conditions, exposure to *L. plantarum* decreased barrier integrity without causing cytotoxicity. Gene expression data from initial studies showed minor (but significant) effects on several immune-related genes. Transcriptomics analysis (RNAseq) comparing healthy and inflamed IECs and Caco-2 cells, and *in vivo* data from the duodenum of healthy humans exposed to *L. plantarum* is ongoing.

The hiPSC-derived IEC model showed more pronounced immune-related responses than the Caco-2 model and is a promising novel *in vitro* model to study effects of bacteria on the intestinal barrier.

Presentation: Oral

718

The OECD vision of a global chemical knowledge base: Towards integration of tools and databases

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The Organisation for Economic Co-operation and Development (OECD) visualises the creation of a Global Chemicals Knowledge Base (GCKB) as a reaction to the existing growth in the volume of data on the safety of chemicals and the rapid spread of databases capturing only some pieces of this information.

The GCKB builds on the current OECD IT tools environment which is based on standardised templates for reporting chemical test summaries (i.e., the OECD Harmonised Templates – OHTs). The OHTs are a key feature of the vision, in which the concept is to have chemical data that is integrated in a structured database (IUCLID) and is interoperable with analytical tools (QSAR Toolbox), tools to build predictive models (AOP-KB), links to verified data sources that include additional data (eChemPortal) and are compatible with third-party databases and model development. Part of this work is also a plan to harmonise ontologies.

The OECD anticipates huge benefits brought by a consolidated global knowledge base. The GCKB could be used by public authorities and other stakeholders for the hazard assessment and management of chemicals or to support chemical safety decision-taking. The information accessible through the GCKB could be also used for other applications such as to support the use of new approach methodologies in regulatory contexts, and to inform predictive toxicology, data mining or machine learning applications.

Presentation: Oral

719

Advancing and enabling human-relevant research in India

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The Centre for Predictive Human Model Systems has been working towards enabling human-relevant research in India for the last four years. Our activities have been targeted at four verticals: education/training, public outreach, policy, and creating a community of human-relevant researchers in India. These verticals are based on the white papers that we created to understand the landscape of non-animal research in India. We have been conducting a monthly webinar series for the last two two years; several Adverse Outcome Pathway workshops across the country that resulted in two AOPs being submitted to the AOPwiki by two Indian scientists. In Oct 2022, we received a grant from European Molecular Biology Organisation (EMBO) to conduct the first-ever India EMBO Lecture Course on Microphysiological systems (MPS) which brought together both the Indian and international communities of researchers working on cutting-edge MPS technologies. We have also recently started a virtual lab series via which undergraduate students will get exposed to a lab working on human-relevant technology in India virtually. Under the policy vertical, we conducted two high-level multistakeholder meetings with the government and regulatory bodies in India towards advancing this field in India. Through a combination of these activities, we are raising awareness for non-animal methods, training the students and researchers in these technologies, creating a pipeline for indigenous development of these technologies in India, connecting the community of researchers, industry personnel, regulators, and government bodies together to develop India as a key player in the area of human-relevant research in the coming decade.

Presentation: Oral

721

Free, open access training and competence assessment tools – No excuses left!

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Professional competence is the corner stone of ensuring appropriate care and welfare of animals used in research and testing. It is built on the basis of high-quality education, training and continued professional development. The tools to develop, assess and maintain competence should be accessible to all those involved in care and use of animals.

Thanks to a funding by the European Parliament, a number of activities are taking place to support high quality, accessible and affordable training for all. These projects cover a wide range of activities with the specific focus on developing open access educational and training tools and resources for a variety of target audience ranging from secondary school to early career scientists, from training providers and trainees to competence assessor.

Modern technologies play an increasingly important part in today's educational delivery. The offered tools will use interactive learning techniques for example those used in open access eModules. These can be accessed through a central user-friendly plat-

form hosted by ETPLAS, the Education and Training Platform for Laboratory Animal Science. Several such tools have already been completed and are available for those interested. The on-going projects include development of further tools with a specific focus on supporting competence assessment – irrespective of where in the world it is needed.

Presentation: Oral

725

Non-technical project summaries to direct funding decisions on research on alternatives

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Transparency should be one of the corner stones of a legal framework under which animals are used in research and testing. Transparency is also one of the three key aims of the legislation in place in the EU today. Not only are detailed annual statistics on animal use published through an open access database, researchers are also required to provide a clear and easily understandable non-technical project summaries of new projects authorised to use live animals. Since July 2021, these non-technical project summaries are also available to anyone interested through an open access EU database, ALURES NTS.

Non-technical project summaries provide better understanding on why and how animals are still used today; the areas of use and the severities related to such uses. But equally, information on how the Three Rs are being implemented in specific projects. Non-technical project summaries can also provide further insight into the type of research activities that are on-going in specific areas, for example related to specific human diseases.

Decisions on research funding for the development of new alternative approaches can use these tools to focus on areas using high numbers of animals, and those resulting in highest severities. Non-technical project summaries can also provide further understanding into the obstacles in replacing animals in these areas. Together with statistical data, we have better tools than ever before to ensure that investments into alternatives are made in areas that can have a real impact on reducing animal numbers and suffering.

Presentation: Oral

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Nonhuman primate research ethics beyond the 3Rs

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Though much work in applied ethics assumes the prominence of formal justice considerations – that like should regarded alike – this remains under appreciated, if not ignored, in the animal science communities. This lies in significant tension with an increasingly common refrain that the scientific use of animals adheres to our highest ethics standards. Though the 3Rs can be supported by a principle of non-maleficence, one that is related to a well-respected principle in applied ethics, our highest ethics standards require much more. This talk will show how formal justice considerations along with our best scientific understanding of nonhuman primate minds nudge us beyond a fixation on non-maleficence, however non-maleficence is understood. It is not uncommon to use humans as the comparison in using formal justice to leverage greater moral regard for other animals, but this talk will show how changes in the regard for research chimpanzees in the US also supports re-seeing our moral obligations to primates who are not great apes. Moreover, changes already afoot in how animals are treated while bred, raised, or used in science are best understood as reflecting a move beyond non-maleficence. Though this talk will argue that those changes are not enough, they require repositioning the 3Rs as supplementary to more core ethics commitments and call for a new approach to animal research ethics.

Presentation: Oral

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"Adverse outcome pathways:" A perfect framework on which to build a thesis

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The AOP framework provides a structure for collecting, organizing and reviewing biological information that is perfectly suited to formulating a solid research project. Central to the AOP framework is the AOP Wiki, a freely available online tool for crowdsourcing biological information. The planning of a nascent research project benefits greatly from a structured search of existing information; the AOP Wiki fields, if extracted as a template, provide a guideline for organizing this information as either key events (KE) or key

event relationships (KER) and captures several dimensions of the information (species, life-stage, sex), and if relevant, any linkage to a known external perturbation (chemical, virus, or other environmental factor). The wiki fields for describing KER also provide a framework for evaluating biological information, including aspects of biological plausibility, empirical evidence, temporal concordance and uncertainties and inconsistencies. Perhaps one of the most powerful advantages of implementing a research topic as an AOP is that the wiki links this information to all other information in the wiki and this advantage only grows with increased participation. To support this approach, the existing model of AOP review by scientific journals could serve as a catalyst and precedent for requiring AOP implementation as a condition of research funding, either by public sources, or through private research foundations. Using the AOP framework as a foundation ensures that the new research builds on the current state of knowledge, avoids duplication, facilitates publication and allows the research to focus discovery in areas that lead to the greatest impact.

Presentation: Oral

729

Using a standardized tool to assess the translational (ir)relevance of animal models

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The high failure rates in drug development have fueled the debate on the translational relevance of animal models of human diseases. The reason for high attrition rates is multifactorial and includes internal and external validity issues. The Framework to Identify Models of Disease (FIMD) is a question-based tool developed to standardize the assessment, validation and comparison of disease models, aiming to evaluate the predictive value of these models based on a translational rationale, which results in a final score that indicates the similarity to the human condition.

The Biomedical Research for the 21st Century (BioMed21) Collaboration designed a pilot study to evaluate the applicability of FIMD to identify animal models with low translational relevance and the usefulness of the results to drive change in animal research funding and the use of animal models.

For the pilot study, sepsis was chosen as indication because of the high failure rates in the clinic and availability of models of differing etiologies. Two models were selected for comparison: the cecal ligation and puncture (CLP) model, considered the gold standard; and the lipopolysaccharide (LPS)-induced model, which is claimed not to adequately represent the disease in humans.

A publication and webinar are planned to disseminate the findings and discuss how FIMD can be applied by different stakeholders to avoid the use of animal models with low translational relevance, promoting the identification and implementation of models more likely to predict the human response instead.

Presentation: Oral

734

Qualification of the devTOX quickPredict assay for regulatory use under the ICH S5(R3) guidelines

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Multiple regulatory agencies have released new guidelines permitting the use of new approach methods (NAMs) in conjunction with or in place of the traditional *in vivo* embryo-fetal development (EFD) studies. In particular, the ICH S5 (R3) guideline defines specific scenarios where qualified NAMs can be used to defer or replace conventional *in vivo* testing. The human pluripotent stem cell-based devTOX quickPredict (devTOXqP) assay predicts a compounds developmental toxicity potential based on changes in ornithine and cystine metabolism and has been used by multiple industries for almost a decade. devTOXqP has an accepted letter of intent with the FDA's Center for Drug Evaluation and Research Biomarker Qualification Program to qualify the assay as a safety biomarker for detecting human developmental toxicity potential *in vitro* at the nonclinical stage of drug development for small molecule drugs as part of a weight-of-evidence assessment as described the ICH S5(R3) guideline, which will enable regulatory use of the assay in the pharmaceutical industry. As part of this qualification, we have evaluated the performance of the assay across a diverse set of 89 pharmaceuticals, including the ICH reference compounds. The assay predicted the developmental toxicity potential of these pharmaceuticals with a balanced accuracy of 85% (83% sensitivity, 88% specificity). The devTOXqP assay provides data on human response and is a necessary addition to protect human health, replace (in certain cases) and reduce animal testing, and to help to reconcile discordant information from the required *in vivo* endpoints.

Presentation: Oral

736

New approaches to comparing potencies and hazards of emerging BPA alternative chemicals

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Many countries worldwide have shifted away from bisphenol A (BPA) due to health concerns, leading manufacturers to increasingly use BPA alternative chemicals. We used global gene expression profiling to compare potencies, assess estrogenicity, and identify potential modes of action of BPA and several alternative chemicals individually and as mixtures.

MCF-7 breast cancer cells were exposed to the chemicals (0.0005-100 µM) for 48 h. TempO-Seq sequencing (BioSpyder Inc.) was used to examine general toxicological effects and estrogen receptor alpha (ERα)-associated transcriptional changes. Benchmark concentration (BMC) analysis was conducted to identify global transcriptomic points of departure (tPOD). ERα activation was evaluated using a published ERα biomarker and an ERα-specific tPOD was also derived. Genes fitting BMC models were subjected to upstream regulator (UR) and canonical pathway analysis in Ingenuity Pathway Analysis.

Potency ranking using global tPODs established bisphenol AF (BPAF) as the most potent chemical tested overall. The ERα tPOD also highlighted BPAF as the most estrogenic chemical tested and the ERα biomarker identified novel ERα activators among data poor alternative chemicals. URs and canonical pathways perturbed by BPA and alternative chemicals revealed modes of action associated with cell cycle control, proliferative signaling, and cancer. Effects of BPA alternatives in mixtures are predicted to be additive due to common molecular targets.

These data suggest that many of the BPA alternative chemicals are ERα activators and alter gene expression in MCF-7 cells. This study provides baseline data to inform read-across and prioritize these chemicals for further assessment.

Presentation: Oral

739

Chronic drug-induced cardiotoxicity assessment using *in vitro* human iPSC-cardiomyocytes and human heart slices: A multi-platform study conducted by the HESI Stem Cell Working Group

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International guidelines (ICHS7) describe assays to detect drug-induced acute delayed ventricular repolarization and proarrhythmic risk, but do not address potential chronic drug-induced changes in electrophysiology, cardiac structure or function. For example, anthracyclines, some tyrosine kinase inhibitors and ion chan-

nel trafficking inhibitors are linked to increased risk for cardiotoxicity over prolonged and cumulative exposure periods. Moreover, the specific mechanisms of cardiotoxicity remain unclear. There is an urgent need to better understand the risk of long-term deterioration of cardiac function. The HESI Cardiac Committee organized and executed multisite studies to assess the effects of prolonged exposure of 12 reference drugs known to affect the human heart. *In vitro* assays with different technologies employing human iPSC-cardiomyocytes in 2D monolayers and 3D microtissues and adult heart slices were used to test 12 drugs chosen to address 4 critical endpoints of cardiac function: energetics; electrophysiology; contractility; cardiac structure. These compounds were tested on 24 different platforms yielding 50 biomarkers related to cardiac structure and function. These biomarkers were monitored for an exposure period of up to 144 hours. This large dataset provides information on the sensitivity of key biomarkers of cardiac structure and function necessary to detect chronic cardiotoxic actions of drugs.

Presentation: Oral

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A path forward for new approach methodologies for developmental immunotoxicity testing

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To date, developmental immunotoxicity (DIT) testing is intimately linked to the use of whole animal studies with inherent limitations in translatability to humans. Moreover, human clinical information on the effects of drugs and other exposures on the developing immune system are scarcely available. Therefore, there is

a critical need for the development of non-animal New Approach Methods (NAMs) for applied and regulatory DIT testing purposes. Due to the complex nature of the effects and partially missing information on interrelationships in the developing immune system, only few NAMs have been developed. None of these have been accepted for regulatory purposes. The "International Working Group on Alternatives to Developmental Immunotoxicity Testing" is identifying and addressing critical knowledge gaps in the field of alternative DIT. Efforts to translate scientific advances into a network of key molecular and biological events, and adverse outcome pathways that can inform regulatory hazard characterization or risk assessment will be discussed. Examples of existing NAMs that are appropriate for DIT testing will be provided. Areas in need of the development of new DIT models and tests will be highlighted through the introduction of a novel conceptual framework to encourage the refinement, development, and validation of NAMs suitable for (high throughput) screening DIT compounds. A future outlook on how ground-breaking innovations and state-of-the art technologies can benefit the switch to alternatives to DIT will be presented. Ultimately, the goal is to develop alternative DIT screening methodologies and test guidelines that can be incorporated into OECD guidance documents.

Presentation: Oral

743

Animal welfare and 3Rs advantages of home-cage monitoring systems

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Home cage monitoring (HCM) can be defined as a technology for continuous and non-invasive collection of behavioural and/or physiological data on animals in an enclosure that provides food, water, social contacts, and environmental enrichment. It applies cameras, sensors, and other devices to collect data on activity, movement, feeding, drinking, and other physiological parameters in undisturbed animals, providing a comprehensive understanding of animal responses to experimental treatments without the impact of handling and the measurement procedures themselves [1], thus benefitting both animal welfare and the reliability of results. Furthermore, HCM systems can detect subtle changes in animal behaviour or physiology that traditional observational methods cannot, monitor animal health and welfare parameters (e.g. body temperature), and allow intervening if an animal shows signs of distress or goes beyond a predefined threshold (e.g. hypothermia). Aside clear Refinement benefits, HCM has also Reduction implications, not only because more data can be obtained without need for additional animals, but also because it reduces handling stress-related variability, a source of experimental "noise", hence requiring smaller sample sizes for detecting a given effect size. This talk will cover how HCM can reduce the impact of routine procedures, based on our research on stress-induced hyperthermia using contactless technology. It will also present our in-house developed low-cost HCM system, and the TEATIME Cost action (http://cost-teatime.org) bringing together researchers to improve animal behaviour research by HCM.

Reference

[1] Blenkuš et al. (2022). doi:10.3390/ani12020177

Presentation: Oral

745

Survey says: How Canadians view the use of animals in post-secondary education & training

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More than 150,000 animals are used in the education and training of individuals in post-secondary institutions in Canada every year. Complete replacement of those animals with fully humane curricula is possible, so why has this not happened? The simple answer – the Canadian public is unaware of the issue. This is no accident; it is a purposeful strategy to make it difficult and often impossible to get any information about animal research in Canada.

The Canadian Council on Animal Care (CCAC) is exempt from the Access to Information Act. Denial of information as experienced in Ontario is cloaked in alleged threats to researchers and their facilities by the animal rights community. Hence, there is little to no public discourse about what happens to research animals in Canadian laboratories.

Featuring the results of a comprehensive survey commissioned by the Animal Alliance of Canada Fund conducted in 2023, this presentation will outline how Canadians view the various issues inherent in the use of animals in post-secondary education and training, such as the use of colony animals, the painful and invasive procedures practiced on them and the issue of pound seizure for sourcing of animals.

The presentation will close with strategies animal protection organizations and scientists can use to bring about greater awareness among the general public, foster a more educated discourse with

curricula developers and convince governments to mandate the funding bodies, CCAC and research facilities to conduct research in an open and transparent manner.

Presentation: Oral

749

EAS-E suite: An integrated platform for new approach methodologies to facilitate hazard, exposure, and risk assessment

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New Approach Methods (NAMs) like *in vitro* bioactivity data, *in vitro-in vivo* extrapolation (IVIVE), and *in silico* approaches (e.g. quantitative structure activity relationships (QSARs)) can be used as part of Integrated Testing Strategies (ITS) to reduce animal use and to address significant data gaps. However, application of NAMs for scientific evaluations and decision-making requires expert judgement, a careful evaluation of the results (Applicability Domain, uncertainty) and it is not always clear how to select the right data in the correct context.

Here we introduce the Exposure And Safety Estimation (EAS-E) Suite, a free on-line platform developed to bridge the gap between evolving scientific research and regulatory assessment challenges. EAS-E Suite facilitates the integration, and application of various curated databases, QSARs, environmental fate, Physiologically Based Kinetic (PBK), exposure and risk estimation models. The platform includes curated, measured physical-chemical properties, environmental biodegradation and toxicokinetic data for > 50,000 chemicals, QSARs for predicting chemical information if measured data are unavailable, and tools such as the PROduction-To-EXposure High Throughput (PROTEX-HT) model to simulate aggregate (far-field and near-field) exposures to humans and ecological receptors. EAS-E Suite provides opportunities to address regulatory challenges for new and existing chemical assessments for ecological and human health objectives. It supports a One Health approach to chemicals management and guides the safe and sustainable production and use of chemicals in society. A case study illustrates how the platform integrates various data sources and knowledge across multiple disciplines to reduce uncertainty, improve chemical management and support decision-making.

Presentation: Oral

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Better animal welfare and better science? – Observations by a former inspector

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For almost a decade, Kathrin Herrmann worked as a federal regulator, inspecting animal experiments in Germany. Kathrin felt that as a veterinarian she should work within the current system to help improve the lives of individual animals used in the name of science. By inspecting numerous animal laboratories and breeding facilities and assessing countless animal research proposals and their scientific outcomes (if they were published), Kathrin became exceedingly aware of the considerable harms involved and the scientific flaws of animal-based research. Alongside her work as an inspector, Kathrin carried out a research project, assessing the use of refinement in practice. She focused on experimental refinements in over 500 animal research applications comprising recovery surgical procedures from around Germany. Her published results show that the evaluated proposals did not incorporate all existing measures to avoid needless suffering; this confirmed the trends found in other reviews of published animal studies from around the world. Considering that almost all animal research proposals are being authorized in the EU, combined with the generally still poor quality of animal studies, so-called competent authorities seem to be unequipped to ensure that only research projects that have a realistic potential to produce benefits, which outweigh the harms inflicted on the animals, are granted licenses. The poor application of refinement methods in laboratories and a malfunctioning regulatory body emphasize that existence of refinement methods does not lead to their application and thus does not lead to improved quality of animal experimentation, let alone to improved translation.

Presentation: Oral

755

The legislative and advocacy road to reform

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It's been a decade since the European Union made history by implementing a landmark ban on animal testing for cosmetics and the sale of newly animal tested cosmetics, establishing itself as the

world's largest cruelty-free beauty market. NGOs including Humane Society International launched global initiatives to extend the EU precedent to other key cosmetic markets, inspiring policy reform in more than a dozen additional countries. These reforms would not have been possible without increased policy convergence and collaboration among NGO and industry stakeholders, including Lush, Unilever, Proctor & Gamble, Avon, L'Oréal and other brands and associations.

Here we present a decade of progress of the legislative and advocacy road to reform in 42 countries that have banned cosmetic animal testing to date, including how we overcame policy challenges, increase capacity building and adapted to meet cultural differences. Furthermore, we will also include an overview of the current efforts legislative efforts to secure robust bans in key beauty markets.

Presentation: Oral

762

High-throughput BMC analysis of global gene expression in human liver spheroids suggests PFAS have additive effects in mixtures

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Per- and polyfluoroalkyl substances (PFAS), so called "forever chemicals", are persistent, widespread contaminants, making exposure to PFAS mixtures a concern. PFAS have long biological half-lives measuring in years and can have serious consequences to health through long-term disruption of biological pathways. The objective of this study was to determine the concentrations of single PFAS and PFAS mixtures that elicit changes that suggest toxicity and determine if the model of concentration addition can provide an accurate estimate of mixture potency. Previous studies from our group have used high-throughput transcriptomic analysis of PFAS exposed primary human liver spheroids to examine effects of PFAS exposure. New data for PFAS-mixture exposures were collected and analyzed along with our previous data using a recent universal transcriptomics pipeline (R-ODAF). BMDExpress3 was used to determine benchmark concentrations (BMC) for all gene expression changes for each PFAS and mixture. The genes with the 25th lowest ranked BMC for each mixture or PFAS were used as their transcriptomic point of departure, indicating toxicity. BMCs were first used to determine potencies of individual PFAS and PFAS mixtures. Mixture BMC predictions were also calculated from component potencies using concentration addition, which sums the potency of each component by their proportion in the mixture. Empirical mixture potencies closely matched those found through concentration addition, suggesting this method may be acceptable for estimating the effects of PFAS mixtures and supporting the concept that PFAS may cause their effects through shared mode(s) of action and likely do not have synergistic or antagonistic interactions.

Presentation: Oral

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Hazard and risk assessment of a new cosmetic ingredient using validated methodologies and new approach methodologies (NAMs): A case study

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Legislations across the globe have banned animal tests of cosmetics and new ingredients. Despite the evolution of alternative methods, not all questions can be addressed using only the validated methods recognized by international institutions. Integration of NAMs and the use of Next Generation Risk Assessment strategies can help us overcome some of these gaps. Here, we employed an Integrated Testing Strategy (ITS) to evaluate key toxicological aspects of a new cosmetic ingredient that has a natural macromolecular sun blocker potential considering its application in facial makeup and sunscreen. The ingredient was classified as "non-irritant" dermal and ocular based on the results of tests following OECD Guidelines 429, 491, and 492. Tier 1 phototoxicity test (OECD 432) resulted in a PIF of 2.7, meaning a possible phototoxic potential. In tier 2, this classification was refuted based on the negative result from the phototoxicity test with a tridimensional skin model (OECD 498). Regarding the genotoxicity potential, despite the negative result in the Ames test (OECD 471), the micronucleus (OECD 487) indicated a genotoxic effect. Further investigation was done using the ToxTracker test (Toxys) which confirmed the non-genotoxic classification. Sensibilization evaluation, in the "2 out of 3" approach, classified the ingredient

as a sensitizer (OECD 442C = positive, OECD 442D = negative, OECD 442E = positive). Nonetheless, GARDskin Dose-response test and Quantitative Risk Assessment 2, allowed the definition of a safe dose. We demonstrated that, by combining NAMs with OECD tests, we improve toxicity prediction and enable the use of new ingredients in cosmetic formulations.

Presentation: Oral

764

On the wild side: Strategies for advancing the 3Rs education of wildlife researchers

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While the 3Rs principles are widely recognized as fundamental guidelines for ensuring ethical and humane treatment of animals in research and teaching, they are still not ubiquitously embraced across all disciplines of life sciences. A case in point is research on free-living animals, which often involves techniques and approaches that are detrimental to animal welfare. And yet, the application of the 3Rs principles in wildlife research has significantly lagged behind that of research on laboratory animal models. Recent studies suggested that awareness among wildlife researchers about the existence of the 3Rs principles is less than 50% and it is, therefore, essential to provide wildlife researchers with adequate training. Since wildlife research differs significantly from research on laboratory animal models, the 3Rs strategies that are established and effective in laboratory settings need to be adjusted to this discipline. However, the availability of 3Rs courses and teaching resources specific to research on free-living animals has been limited. This presentation will introduce several educational resources that we developed, including an informational website, instructional videos, guidelines, and a course curriculum suitable for undergraduate degrees in wildlife biology, ecology, or species conservation. We will also provide some insights and practical guidance for educators, researchers, and policymakers interested in promoting responsible animal use in wildlife research.

Presentation: Oral

766

Transforming students' minds and hearts through teaching innovative, humane science and bioethics

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Major deficiencies in scientific rigor of animal experiments have become increasingly apparent, ultimately limiting both the reproducibility and the translatability of animal experiments to human settings. Taking into consideration insurmountable interspecies differences, solely refining animal studies will not be sufficient to advance human healthcare. Consequently, Kathrin Herrmann developed a research and educational program at the Johns Hopkins Center for Alternatives to Animal Testing (CAAT) that critically appraises current animal use practices in science and scrutinizes both animal and non-animal models regarding their quality and validity with the goal being improvement of science in general. At JHU, Kathrin has been teaching a course on humane science that covers how to thoroughly search the scientific literature on human-relevant research methods and how to apply innovative, cutting-edge animal-free approaches in science. In addition, she offers a course addressing bioethics and particularly animal ethics issues in science. These courses are rounded out by a monthly, free of charge webinar series for early career and established biomedical scientists on the latest developments in scientific methods as well as by workshops, tailored to the needs of scientists working or wanting to work with animal-free New Approach Methodologies (NAMs). Besides teaching the latest scientific advancements, these courses and training modules help participants to improve their critical thinking skills and thus have the potential to open hearts and minds and to reconsider existing belief systems. All these things are a prerequisite for accelerating the transition towards humane and human-relevant research practices and therefore to better healthcare for all.

Presentation: Oral

768

Accounting for *in vitro* and *in vivo* kinetics in quantitative *in vitro* to *in vivo* extrapolations of organophosphate pesticide toxicity

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The toxicity of organophosphate pesticides has been widely studied in animal models. However, next generation risk assessment requires the characterization of the risk of pesticide exposure using non-animal test methods. In this study, we developed physiologically based kinetic (PBK) models parameterised using *in silico* and *in vitro*-derived absorption, distribution, metabolism and excretion (ADME) data to extrapolate *in vitro* neurotoxic effect concentrations to rat and human bioequivalent oral doses, a process referred to as quantitative *in vitro-in vivo* extrapolation (QIVIVE). The PBK model was developed for chlorpyrifos, diazinon, fenitrothion, profenofos, chlorfenvinfos, and their respective bioactive metabolites and evaluated using available toxicokinetic data obtained from rat studies. The model was subsequently translated to simulate human tissue concentrations of the bioactive metabolites using a population-based model. *In vitro* acetyl choline esterase (AChE) inhibition data from blood from different donors as well as human neuroprogenitor test (hNPT) data were subsequently used to extrapolate *in vitro* effect concentrations to *in vivo* bioequivalent doses. Tissue simulations and predicted points of departure (PODs) were generally within a 10-fold of observed *in vivo* plasma concentrations and regulatory PODs, respectively. Even though free concentrations *in vitro* varied significantly between chemicals and *in vitro* toxicity assays, accounting for *in vitro* kinetics (e.g., serum constituent and well plate plastic binding), using partition models, had only a minor effect on QIVIVE outcome. This PBK-based QIVIVE approach allowed us to define chemical specific assessment factors to account for interspecies and interindividual differences in risk assessment.

Presentation: Oral

769

Cleaning house – Doing away with outdated practices in sample size estimation

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With the reproducibility crisis hitting the field of preclinical medicine there has been a renewed focus on proper experimental design. A key question concerns how many animals to use in an experiment. Use too few and the science suffers; use too many and we are irresponsibly wasting animal lives. Trying to create properly powered studies can be difficult however, and consequently some old, simpler, methods have been dusted off and put to use.

An alternative method that is seeing a revival is Roger Mead's Resource Equation. Although taught in many textbooks, this method has no actual statistical basis. It was never even conceived as a method for obtaining sample sizes. It will recommend inappropriately small experiments, consistently leading to underpowered studies. Together with similar methods, based on equally faulty logic, these methods contribute to, rather than address, the reproducibility crisis.

Software-aided methods for power analysis will outperform the Resource Equation and its ilk at every turn. We can demonstrate this, empirically, using computer simulations and data from properly conducted animal studies. The Resource Equation will only work in discovering obvious effects, since it is a solely luck-based strategy.

We need to stop using these methods for dimensioning our studies. Guesswork should not be the foundation for important studies. It is both a waste of animal lives and damaging to science. It is time to do away with old practices, it is time to update our textbooks, and it is time to embrace new and better methods.

Presentation: Oral

770

A new documentary film series on humane innovations in veterinary education

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A documentary film series has been produced by InterNICHE to explore humane innovations and replacement of harmful animal use in veterinary education. Titled "DVM: Training the Animal Doctor", the series of 4 episodes follows the trajectory of a veterinary degree and shows how teaching objectives can be met ethically and effectively through the use of humane tools and approaches. These include virtual laboratories, advanced synthetic mannekins, ethically sourced cadavers from client donation programs, and clinical learning opportunities with animal patients. The series explores an on-going transition that reflects a growing commitment to best practice, a recognition of the advantages of "alternatives", and an appreciation of the need for efficient methods that can meet the demand for competence upon graduation. It showcases departments from across the world that are at the forefront of progressive change. Educators, students and producers share through interview their experience of the development and implementation of humane methods, with a focus on knowledge and skills acquisition. Demonstrations of these tools, and student practical classes employing them, are also featured widely. From the series, a diversity of additional video material has been derived. With translation into over 20 languages, these resources have ongoing distribution to heads of department, administrators, students, and campaigners. They are also available for inclusion in training materials. By demonstrating the successes of innovation in diverse contexts, and the feasibility of full replacement across all disciplines, the film series and its associated resources can support new transitions and encourage further enhancement of existing humane education.

Presentation: Oral

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An advanced *in vitro* model of human blood-brain barrier and its interaction with brain cancer neurospheres

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The blood-brain barrier (BBB) plays a vital role in regulating the haemostasis of the brain but serves as the major obstacle that impedes the efficient delivery of drugs. Therefore, developing novel therapeutics that can cross the BBB is crucial in improving the treatment efficacy of brain diseases. Modelling BBB *in vitro* will thus facilitate the development of brain therapeutics and enable mechanistic investigation compared to *in vivo* models. This study aims to establish *in vitro* BBB models to further drug delivery investigation. Here we used the Quasi Vivo® fluidic system to establish the *in vitro* BBB culture under dynamic conditions and compare their permeability to the static *in vitro* BBB models. Human brain microvascular endothelial cells, astrocytes, and pericytes were seeded on the transwell inserts. The permeability of the setup was measured by the trans-endothelial electrical resistance (TEER) and fluorescent dextran extravasation. We also modelled the BBB disruption using both chemical and physical methods. In addition, we were able to co-culture the dynamic BBB setup with human brain cancer neurospheres and studied the paracrine effects between BBB and cancer cells. Overall, this study demonstrated that the dynamic *in vitro* BBB system is a versatile tool for developing efficient therapy for brain diseases.

Presentation: Oral

772

Caring for science: CIRS-LAS for a constructive error culture in lab animal science

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Suppose we openly talked about failed experiments or even mistakes – what would happen?

Talking about critical incidents or unforeseen events leads to open minded discussions in the community of lab animal science.

By avoiding repetition of unsuccessful experiments, the number of laboratory animals and the severity of animal experiments can be reduced.

Opportunities and limitations of an error reporting culture as well as pitfalls in the introduction of the global reporting system CIRS-LAS will be presented (Bischoff et al., 2018). How does it work? At CIRS-LAS.org, anyone can easily and anonymously submit a case report within a few minutes (Enkelmann and Bischoff, 2020). For this purpose, keywords are requested that enable a later search in the database, as well as information on the animal species, the background of the experiment or general information, a short description of the incident and a classification in a subject area. Applied refinement strategies can be mentioned, as well as suspected reasons and possible solutions. Currently, more than 300 people are already using the database. More than 50 case reports have already been uploaded and form the basis for learning from mistakes. Now is the time to take the next step: Make your experience visible by sharing it worldwide via CIRS-LAS.org – with the responsibility for robust research and animal welfare.

Presentation: Oral

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Integrating transcriptomics, quantitative adverse outcome pathways, *in vitro* and *in vivo* kinetics to predict amiodarone toxicity

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Amiodarone is a highly lipophilic drug used to treat arrhythmia. It is known to induce repeat-dose toxicity in the liver and the brain of patients. As such, it is a suitable chemical to assess whether and how new approach methodologies (NAMs) can be used to identify and quantify the health hazards associated with its exposure. In a first study, amiodarone was exposed to the human hepatoma cell line, HepaRG, to assess its potency to perturb key events along the adverse outcome pathway (AOP) for steatosis. Key events included steatotic gene expression changes and triglyceride accumulation assessed using high content imaging. Toxicokinetic-toxicodynamic (TK-TD) modelling was used to construct a quantitative AOP for steatosis. In a second study, we exposed human induced pluripotent stem cells (hiPSCs)-derived BrainSpheres to amiodarone daily for 7 days. Our transcriptomics analysis revealed that amiodarone altered lipid metabolism gene expression at lower concentrations than in the hepatocytes, once differences in *in vitro* distribution kinetics between the two cell models were accounted for. In the third study, a probabilistic physiologically based kinetic (PBK) model for amiodarone was constructed, parameterized using *in vitro* and *in silico*-derived kinetic data, evaluated against rat experimental data in literature, and used to extrapolate *in vitro* hepatotoxic and neurotoxic concentrations to human bioequivalent oral doses, an approach referred to as quantitative *in vitro-in vivo* extrapolation (QIVIVE). The model predicted significantly higher liver than brain levels of amiodarone after repeat dosing and thus a greater risk for hepatotoxicity compared to neurotoxicity.

Presentation: Oral

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Cytotoxic and molecular effects of soil extracts from the Agbogbloshie electronic waste site on the rainbow trout RTgill-W1 and human Caco-2 cell lines

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Electronic-waste (E-waste) sites are notoriously contaminated with complex chemical mixtures thus challenging environmental monitoring, management, and remediation activities. Since E-waste sites are typically situated in under-resourced regions, there are increasing demands for New Approach Methods (NAMs) to reduce the use of whole animal-based tests, and address resource limitation and ethical issues. The objectives of this study were to: A) characterize cytotoxic effects of soil extracts on the rainbow trout RTgill-W1 and human Caco-2 cell lines; B) measure gene expression changes in both cell lines to derive transcriptomic points of departure (tPODs); and C) compare the cytotoxicity and molecular results between cell lines to determine whether tPODs are protective of *in vitro* LC50 values, while also providing clues into possible toxic mechanisms of action. Extracts were prepared from 35 soil samples collected at 35 sites near the Agbogbloshie E-waste site (Accra, Ghana), including upstream (6), downstream (2), community (3), trade site (8), dump site (13), and burn site (3). Both cell lines were exposed to extracts at concentrations equivalent to 9.38, 4.69, 2.34, 1.17, 0.59, and 0.29 mg dry weight of extract (eQsed)/ml. Cytotoxicity results showed that trade site #8 was the most cytotoxic site for both cell lines, with Caco-2 cells being less sensitive than RTGill-W1 cells. Molecular responses will be measured by ultraplex RNA sequencing technology, from which tPODs will be calculated. This work is expected to support ongoing efforts in establishing efficient alternative testing strategies for environmental monitoring and remediation activities in contaminated sites at resource-limited locations.

Presentation: Oral

776

How far should refinement go?

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Ethical science mandates providing animals in research with a "good life" rather than merely reducing their negative experiences. How far must refinement go to make any meaningful improvements to animal welfare? This presentation will examine the impacts of specific refinements to animal welfare and critically evaluate the number and type of refinements that may be required to achieve a good life for animals in research. The feasibility and likelihood of implementing these refinements in the real world – when considering barriers that are material (e.g., budget, space, time) and immaterial (e.g., institutional culture, psychology of change) – will be discussed.

Presentation: Oral

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Regulatory considerations on new approach methodologies for developmental immunotoxicity

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Developmental Immunotoxicity is a rapidly evolving field in both methods and advancing knowledge. As with other areas of chemical safety evaluation, there are multiple reasons to move toward New Approach Methods (NAMS). These include improved relevance to human biology, reduced use of animals and increased capacity for testing. Developmental Immunotoxicity has a real opportunity to advance the science and risk assessment process by focusing on the development of NAMS as the science evolves. The relevance and fit for purpose aspects of any NAMS is important to consider during development, qualification and implementation. NAMS that helps elucidate mechanism of action and identify potential hazards may be useful to help clarify animal and epidemiological data suggestive of a risk. While such data are potentially useful, regulatory decisions must depend on data based on appropriately qualified methods to ensure decisions are dependable and appropriately advance public health. To that end, a qualification program assesses NAMS for specific context of use, the regulatory question it addresses, relevance to human developmental immunotoxicity including specificity and sensitivity and repeatability across laboratories and researchers. Qualification of a NAMS for any endpoint including developmental immunotoxicity is not a simple or quick task and involves the use of reference and control compounds. It takes planning, flawless execution and cross laboratory collaboration. The field of developmental immunotoxicity and its evolving knowledge and methods has the opportunity to contribute in the area of NAMS development to advance public health safety.

Presentation: Oral

778

Advanced *in vitro* models for nephrotoxicity risk assessment

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Nephrotoxicity is a major cause of kidney disease, a common reason for drug development failure, and a contemporary challenge for chemical risk assessment in humans. Historically, tools for nephrotoxicity assessment consisted of 2D monocultures of undifferentiated human- or animal-derived renal epithelial cells and mammalian animal models. These systems often fail to predict nephrotoxicity due to their limited ability to recapitulate fundamental aspects of human kidney structure and function. Renal injury accounts for ~20% of drug candidate attrition during phase 3 human studies but just 2% during pre-clinical studies. Proximal tubule (PT) cells are the most frequent site of damage as they transport and metabolize xenobiotics, leading to intracellular accumulation of reactive metabolites, which can impair PT solute reabsorption and disrupt essential nutrient homeostasis, causing negative health effects.

In vitro systems that mimic human PT physiology hold the potential to model and predict nephrotoxicity, toxicodynamics, and species differences. In this presentation, we introduce two types of advanced *in vitro* systems that we developed to address unique aspects of nephrotoxicity assessment: (1) screening level system – designed to create interpretive context for decision making by efficient survey of chemical structures, exposure dose/time, and reversibility – consisting of 3D microtissues of human PT cells that demonstrate enhanced differentiated longevity and sensitivity to nephrotoxic compounds compared to 2D cultures; (2) microphysiological system (MPS) that integrates microfluidics with tissue engineering to mimic *in vivo* dynamics and functions. Both types of advanced *in vitro* models are required to address contemporary challenges of NAMs-based risk assessments.

Presentation: Oral

779

Gaps and considerations in oversight and research limitations in use of human nonhuman animal chimeras

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The use of human-nonhuman animal chimeras in medical research raises important scientific, ethical, and legal questions about the validity and oversight of this experimental approach. This presentation examines the current state of chimeric research, as defined by the integration of human stem cells with nonhuman animal embryos. The outcomes of human-nonhuman animal chimeric studies purportedly provide insight into stem cell therapy, rehabilitation science, and xenotransplantation. From the viewpoint of research protections, it not only introduces new considerations into the current validity of monitoring the pain and suffering of animals used in research, but also reignites debate as to the biomedical enterprise's ability to 1) self-regulate, 2) determine limitations of nonhuman-animal-based methodology, and 3) develop oversight to replace and reconsider animal use in translational science. We present an overview of the guidelines and regulations from countries and international research bodies that currently govern chimeric research and describe the gaps in oversight and broader discourse. We highlight potential applications of chimeric research and their possible intersections with existing regulatory and legal frameworks. Finally, we describe human-specific nonanimal methods that can reduce the reliance of nonhuman animal *in vivo* experiments and reflect on historical pitfalls in regulation of genetic modified animals to help forge a more human-relevant path forward using human stem cell science.

Presentation: Oral

780

Comparing transcriptomic, metabolic and behavioural points of departure in zebrafish larvae exposed to a diverse suite of toxicants

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Transcriptomic dose-response modelling (TDRM) is a promising quantitative approach for characterizing hazard in chemical risk assessment. Evidence suggests that TDRM can generate transcriptomic points of departure (tPODs) from short-term exposure studies that are protective estimates of long-term toxicity, thereby significantly reducing the need for traditional long-term animal exposure studies. However, the majority of this evidence comes from adult rodent models. Environment and Climate Change Canada is currently investigating the use of TDRM in a variety of ecologically relevant animal alternatives, including fish embryo-larval models (embryos/larvae that are incapable of independent feeding are not regulated as animals in many jurisdictions, including Canada). Here, we present the findings from an ongoing study that compares tPODs to metabolic, behavioural and overt toxicity endpoints in zebrafish larvae exposed to a diverse suite of 30 well-characterized toxicants. Gene expression was measured using Illumina whole-transcriptome sequencing or Qiagen 3'-UPXome sequencing. TDRM was conducted using BMDExpress software and tPODs were determined using pathway-based and pathway-agnostic approaches. Metabolic expenditure was determined using an Alamar Blue assay. Swim behavior was recorded over alternating light and dark periods using a ZebraBox tracking system. Metabolism, behaviour and overt toxicity points of departure (POD) were determined using the rcurvep R library. For each compound we compared the transcriptomic, metabolism, behavioral and overt toxicity PODs determined in zebrafish larvae to traditionally derived PODs in adult fish reported in the literature. This study will provide important information for characterizing the relationship between tPODs and traditional toxicity endpoints used for hazard assessment in fish.

Presentation: Oral

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High throughput cardiac ischemia model human-on-a-chip platform

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Hesperos, Inc. aims to minimize animal testing to improve compound testing efficiency in preclinical trials, reduce late-stage failure and reduce animal testing by utilizing Human-on-a-Chip technology with iPSC-derived human tissues coupled to bioMEMS devices to investigate drug compound efficacy, toxicity, and mechanism of action. Towards this goal, a bioMEMS Human-on-a-Chip system consisting of an iPSC derived cardiac construct was developed to study ischemia-induced damage to the heart via mechanical and electrical functional readouts and to test candidate drugs in a high throughput setting utilizing a multi-organ microfluidic system. A custom environmental control chamber equipped with set point control for gas concentrations and *in situ* air and medium oxygen measurement was developed to dynamically drive environmental gas concentration. An integrated high-throughput *in situ* amplifier system was designed to measure and stimulate 256 electrodes across a range of frequencies to measure the minimum interspike interval (mISI, analogue of QT interval), beat frequency and conduction velocity (CV). To validate the system for use as a phenotypic assay, CV and mISI were characterized under treatments from combination dosing of ivabradine and tolbutamide (reduction in pacing frequency and dose dependent increase in mISI) and E4031 (increase in mISI). Ischemic damage at an environmental oxygen content of 0.1-0.25% reduced CV by more than 80%. Pre-treatment with the candidate drug ZP1609 ameliorated the reduction of CV by 20%. The output of this study was a high throughput measurement platform to study ischemic cardiac damage and interventions utilizing clinically relevant functional readouts without the need for animal models.

Presentation: Oral

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Prioritising comparisons can decrease animal use

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Background: In many animal experiments multiple groups are used. These experimental groups can ensue from different treatments or different kind of animals (e.g., immune-compromised or healthy). Often multiple comparisons between groups are made.

Aim: Investigating how much prioritizing the order of conducting comparisons can decrease use of animals

Methods: We consider a real case study with four groups: control, (known) active substance, enhancer under investigation, combination of active substance and enhancer under investigation. We present a sensible order of prioritization that was in line with the research goals of the researcher. Then we compare the fixed sequence testing procedure corresponding to this prioritization with the commonly used Tukey's method for multiple comparisons.

Results: In our case study, Tukey's method resulted in 72 animals and fixed sequence testing in 40 animals

Conclusion: Considering upfront whether a prioritization in the comparisons of interest is sensible and applying fixed-sequence testing according to the prioritization, may reduce the use of animals.

Presentation: Oral

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Spoiled milk? Replacing animal-derived blocking buffers in immunoassays

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Since its introduction in the late 1970s, Western blotting has become the most widely used immunoassay across the life sciences. While technology has improved significantly with sophisticated imaging equipment, Western blotting protocols have not changed much despite their role in the reproducibility crisis – blamed primarily on poor specificity and sensitivity of animal-derived antibodies. However, blocking/antibody incubation buffers often exacerbate the situation, for example, with high background, non-spe-

cific binding, and epitope masking. Non-fat dry milk and bovine serum albumin are the most popular blocking/antibody incubation buffers – which often require empirical validation by the end-user for each antibody-antigen pair. Our goal was to evaluate the feasibility of replacing animal-derived components in immunoassays using a range of "milk" products derived from soy, pea, oat, almond, brown rice, hemp, coconut, cashew, and pumpkin seeds. We evaluated their performance in Western blotting under varying conditions such as epitopes (including phosphoproteins), antibody isotypes and clonality, antigen source, blotting membranes, and detection methods. Our data indicate that plant milks can elicit robust sensitivity with little to no background with only five minutes of blocking at concentrations as low as 1%, even with phosphoprotein targets. Several plant milks also displayed exceptional performance in immunofluorescence assays. We have discovered a set of versatile, easily accessible, cost-effective, environmentally-friendly, and cruelty-free blocking buffers – with unsurpassed functionality in comparison to their animal-derived counterparts – to advance scientific discoveries while replacing animal-derived reagents in the life sciences.

Presentation: Oral

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Hepatotoxicity-in-a-dish: Microphysiological system to model drug induced liver injury

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Drug induced liver injury (DILI) remains the most common cause of acute liver failure resulting in high drug attrition rates. The gold-standard legacy animal models such as rats, dogs, and monkeys display poor human DILI concordance rates – underscoring the need for new approach methods better able to predict human hepatotoxicity, especially through adverse outcome pathways (AOPs) that elucidate mechanisms of toxicity. The overarching goal of our project was to develop and validate a novel, human hepatic microphysiological system (MPS) that can capture physiologically relevant fibrosis, steatosis, and cholestasis –the three key apical endpoints of DILI. We designed a multicellular, 3D-bioprinted human liver tissue model comprising hepatocytes, stellate cells, cholangiocytes, macrophages, endothelial cells, and lymphocytes – engineerable in defined cellular cytoarchitecture to capture the versatility of DILI apical endpoints. To characterize our MPS, we selected AOP 38 (fibrosis), AOP 34 (steatosis), and AOP 27 (cholestasis) – with diverse key events at the cellular, tissue, and organ levels using a broad range of reference drugs known to cause DILI. Here, we present data from our hepatic fibrosis model validated using AOP 38 key events leading to liver fibrosis (collagen accumulation/changes in extracellular composition and decreased liver function). Our MPS can capture dose- and time-dependent development of liver fibrosis – at or below human plasma Cmax for pro-fibrotic reference drugs in as short as three days of repeated dose exposure. Our versatile MPS can be used in integrated approaches to bridge the preclinical-to-clinical translational gap in DILI prediction and liver disease modelling.

Presentation: Oral

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Alveoli-in-a-dish: 3D-bioprinted human lung tissue model for inhalation toxicity

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Animal models remain the primary method of inhalation toxicity testing for hazard characterization and risk assessment of chemicals posing a threat to the respiratory tract. Given the time and cost of animal experiments and limited translational relevance, there has been a tremendous need to develop human-relevant approaches that can more closely capture human lung pathobiology, especially predictive toxicology approaches that elucidate mechanisms of toxicity through adverse outcome pathways (AOPs). Our goal was to develop a novel microphysiological system (MPS), Alveoli-in-a-Dish, that can recapitulate key features of repeated dose inhalation toxicity *in vitro*. Our Alveoli-in-a-Dish is a complex, multicellular, 3D-bioprinted human lung tissue model comprising type I and II pneumocytes, fibroblasts, endothelial cells, and lymphocytes arranged in defined proportions and 3D cytoarchitecture. To characterize our MPS, we selected AOP 173 that describes inflammation-mediated pulmonary fibrosis applicable to a broad range of chemicals, and we investigated multiple key events at the cellular, tissue, and organ level using reference drugs and chemicals known to cause lung fibrosis. Promising preliminary data indicate that our physiologically relevant MPS can reliably capture – in a dose and time-dependent manner (as early as three days) – multiple key events of AOP 173, including increased proinflammatory mediators, loss of epithelial/capillary membrane integrity, fibroblast/myofibroblast proliferation, and accumulation of collagen leading to lung fibrosis. With engineerable versatility, our reproducible and

standardizable MPS can be used in integrated approaches to bridge the translational gap in acute and repeated-dose inhalation toxicity testing as well as pulmonary disease modelling.

Presentation: Oral

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Animal-free methods in life sciences and healthcare – Successes and challenges

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Based on our long-term goal of replacing the use of animals in research and development with equal or improved non animal methods (NAMs), their acceptance is essential. With Responsibility as a central fourth pillar alongside Replace, Reduce, Refine, we have launched our company-wide 4Rs program. In addition to sound global governance that goes beyond local laws where necessary, the development of NAMs in our Life Science and Healthcare businesses is a key driver for achieving the goals we have set. Although we have implemented several successful projects to replace animal testing in safety testing and diagnostic products, there are challenges to achieve this across all areas of our diverse business.

Despite historical and still significant challenges, we are seeing movements in regulatory agencies towards accepting NAMs that open doors for pharma research to advance the gradual replacement of animal testing. For example, the US Pharmacopeia acceptance of an *in vitro* alternative to replace the class VI testing in rabbits, the FDA modernization act 2.0 which allows for consideration of NAMs and advanced regulations in EU that prohibit the use of animals where accepted animal-free alternative exists. Furthermore, we are seeing an increased request and demand for NAMs in our Life Science customer base, we will use this momentum to drive our development in the NAM space. We believe that proactive exchange and transparency about successes and – perhaps even more so – failures between scientists in industry and academia are critical to the gradual replacement of animal testing methods.

Presentation: Oral

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In vitro cell models for identifying potential metabolic disrupting chemicals

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The prevalence of metabolic diseases including type 2 diabetes, non-alcoholic liver disease and obesity are on the rise worldwide. High fat diet, genetics and a sedentary lifestyle are known contributors to the disease. However, it is now clear that other factors are involved. Exposure to pollutants, were proposed to also play a crucial role. Although it is now accepted that chemical exposures may increase the risk of developing diabetes, obesity and fatty liver, the assays to screen chemicals for these effects are lacking. Our lab focussed on two important endocrine and metabolic tissues, the adipose and the liver. We investigated the effects of several groups of chemicals including bisphenols and per- and polyfluoroalkyl substances on primary human preadipocytes and human primary liver cell spheroids, coupled with high throughput transcriptomics. Human primary preadipocytes were exposed to bisphenols during differentiation and as mature adipocytes. At day 14, RNA was extracted and RNA-seq was conducted to assess global transcriptomic changes. For the liver, human primary liver cell spheroids were treated with the chemicals for 24 hours or ten days. Temo-seq was used as a platform to investigate changes in gene expression. Our data show that chemicals can disrupt lipid metabolism, affect adipokine release and alter the function of these two important metabolic tissues. Further, our data show that these models can be used for ranking the potency of chemical and identify plausible mode of action.

Presentation: Oral

795

Pharma actions to push the boundaries of the Three Rs in non-human primate use

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Non-human primates (NHP) are widely used in biomedical research and for regulatory testing which raises serious ethical concerns and has resulted in growing societal pressure to move towards alternative research methods.

The pharma industry is working hard to reduce and refine NHP use as much as possible. However, NHPs are often considered the only relevant species for nonclinical safety studies required for regulatory submissions. To obtain a better insight and visibility of the impact of the NHP use in the pharma industry, EFPIA surveyed its members in 2022 & 2023.

The industry is fostering efficient development of safe and effective innovative health technologies and collaborating in projects which contribute to the development, validation and uptake of alternative approaches which could provide in an efficient manner similar or higher levels of information or products of higher quality than those obtained with procedures using animals, but which either use fewer animals or do not involve the use of animals. A partnership of multidisciplinary public, private stakeholders as well as regulatory agencies can help to address the scientific and regulatory challenges and to accelerate the development and use of effective new approaches and non-animal technologies.

There is room for more scientific research on alternatives that would increase confidence in translatability for humans, and into more robust human *in vitro* models (organ on a chip, 3D models). Furthermore, the development of guidance and wider acceptance from regulatory authorities is essential including global alignment amongst regulators.

Presentation: Oral

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Advancing acceptance of NAMs for regulatory testing of medicinal products in the EU

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The European Medicines Agency (EMA) has a long-standing commitment towards the application of the principles of Replacement, Reduction and Refinement (3Rs). This is driven by the requirements of Directive 2010/63/EU, as well as by the crucial need for better tools to predict quality, safety and efficacy of new medicinal products.

EMA's regulatory science strategy 2025, clearly recommends the leverage and qualification of 3R testing approaches or Novel Approach Methodologies (NAMs). It recognises the need for discussion on and definition of regulatory acceptance criteria (e.g. context of use, endpoints and reference compounds) in order to promote regulatory acceptance of NAMs. For this engagement with stakeholders is seen as instrumental.

Recently, a new 3Rs Working Party (3RsWP) has been set up as the official 3Rs hub for at the EMA. The 3RsWP is initiating a broad set of activities dedicated to the qualification of NAMs or 3R testing approaches. These include for instance the organisation of workshops on microphysiological systems, including organ-on-chip with a specific focus towards method qualification, the definition of regulatory acceptance criteria for organ-on-chip technologies for specific contexts of use and the initiation of an international regulatory conversation in order to harmonise views and acceptance criteria.

Specific challenges and opportunities related to the regulatory acceptance of NAMs for the testing of medicinal products will be addressed in this presentation. Moreover, the actions of 3RsWP to foster further uptake of NAMs, as detailed in the 3-year workplan, will be presented.

Presentation: Oral

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Application of multi-organ-chips as NAMs in risk assessment

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In principal risk assessment is based predominantly on evaluations carried out on individual substances. However, humans are exposed to a wide variety of substances with potential adverse effects of the interactions between those substances when present simultaneously in any kind of mixture. For instance, substances may act jointly in a way that the overall level of toxicity is affected or may produce combination effects that are larger than the effects of each substance applied separately. An interesting strategy to address this matter is through the use of microfluidic microphysiological system (MPS) enabling to emulate complex human or animal biology *in vitro*. In particular, the HUMIMIC Multi-Organ-Chip platform is capable of maintaining and culturing miniaturised organs emulating the biological function of their respective full-size counterparts over long periods. Major biological features such as pulsatile fluid flow, efficient nutrition, and physiological tissue-to-fluid and tissue-to-tissue ratios can be incorporated. Moreover, the platform supports the development of a range of testing needs, including repeated dose testing up to at least 28 days. Selected case studies of using (Multi)-Organ-on-a-Chip solutions for substance evaluation will be presented. The system's robustness as well as its capacity to provide *in vivo* relevant information about exposure scenario-dependent changes in bioavailability will be evaluated. Lastly, the concept of individualized risk assessment through the usage of personalized MPS systems will be discussed.

Presentation: Oral

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Enhancing science education by ending high school dissection

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High school biology courses at some educational institutions include the practice of animal dissection. In other locations, humane traditions such as observational studies of animals in their natural habitat, and the use of innovative, non-animal tools, are standard practice. In some countries, dissection at the secondary educational level is banned by law. This presentation explores the resistance to changing teaching methods and explains the importance of focusing on and reviewing teaching objectives so as to enhance students' acquisition of knowledge, skills and attitudes. It also addresses the broader themes of critical thinking, emotional and ethical literacy, and responsibility, along with the concept of the hidden curriculum. The feasibility and desirability of an ethical education are demonstrated through its pedagogical, scientific, ethical, economic and environmental advantages. Examples of suitable tools and their implementation are given, along with suggestions of how to facilitate the transition to a fully humane and more effective and relevant education.

Presentation: Oral

Poster Abstracts

3

The effects of individual housing on nonhuman primates: A review

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Despite serious scientific and ethical considerations, approximately 200,000 nonhuman primates are used in biomedical research worldwide each year, including 68,257 of them in the U.S. and 4,805 in Canada in 2019. In 1985, U.S. Congress amended the federal Animal Welfare Act to mandate standards to promote the psychological well-being of primates in laboratories, including the introduction of social-housing requirements. Almost 40 years later, thousands of primates are still housed alone in the U.S. Individually housed primates are restricted from engaging in their natural social behavior and, compared to monkeys who are socially housed, have reduced physical fitness and welfare, altered immune response, and increased stress. Our research reviews the evidence of the psychological and physiological effects of the individual housing of primates. We also present data on primate housing conditions and how they were reported in recent publications across research domains and discuss the impact of housing on data included in these publications. We find evidence that individual housing of primates is still common practice, likely introducing unwanted variables into the data, reducing reliability and validity, and seriously compromising the value of the results. Singly housed primates show signs of overall poor psychological and physiological welfare (e.g., depression-like behavior or stereotypic behavior). We thus highlight the importance of conducting a meta-analysis to investigate the potential long-term effects of housing conditions on primate health. These findings also suggest that research communities need to evaluate findings from any studies that rely on non-socially housed primates more critically.

Presentation: Poster

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Better science, fewer animals: Catalyzing NIH grant-making to improve biomedical research and meet societal goals

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Animal models are currently the "gold standard" in biomedical research. However, new approaches that do not involve the use of non-human animals are evolving to address the public health and medical challenges where animal models are less well suited. There is a clear societal need to encourage such efforts, and there is widespread support to move away from animal-based research by the American public.

The National Institutes of Health funds the majority of biomedical research in the United States and should be a key player in developing new methods. There have been numerous bills introduced before the US Congress that seek to change the way that NIH allocates its resources, with an emphasis on increasing funding for alternatives. To date, none of these bills have advanced in either the US House of Representatives or the Senate.

This article examines how the NIH could utilize the policy options available under its current laws and regulations to move toward a research environment that puts greater value on alternatives and, at the same time, moves away from animal models as the gold standard. The major advantages of this approach is that it can be implemented without changing current laws and regulations, is relatively straightforward, and can be executed relatively quickly. If adopted, these policy options have the potential to create a much-needed paradigm shift that will improve scientific research while, at the same time, responding to the societal desire to use fewer animals in the biomedical arena.

Presentation: Poster

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Applying machine-learning approaches to identify key genes associated with drug-induced cholestasis

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Background and objectives: Due to its complexity, early detection of drug-induced cholestasis (DIC) during drug development remains challenging. Preclinical animal studies often fail to detect DIC in humans mainly due to interspecies differences. Recently, toxicogenomics *in vitro* assays, especially based on human liver cells, have become a more convenient and practical approach for the prediction of human-relevant DIC. Over the past decade, the established large-scale databases, combined with machine-learning (ML) approaches, provide the opportunity to identify transcriptome signatures of DIC. In the present study, we leveraged the publicly available database, Open TG-GATEs, for the identification of transcriptomic signatures of DIC.

Material and methods: We retrieved toxicogenomics data derived from cultured primary human and rat hepatocytes following exposure to 9 cholestatic compounds and 9 non-cholestatic compounds. Transcriptome profiles were measured at two time points following single exposure to a given compound at three dosages with two biological replicates. Due to the mechanistic complexity of DIC, the model cholestatic compounds were selected because of their potential to cause cholestatic hepatotoxicity through diverse toxic mechanisms. Several supervised ML approaches, including Random Forest, Support Vector Machine and Logistic Regression, were applied to the human liver TG-GATEs dataset to develop a prediction model.

Results: We identified a signature consisting of 20 genes that predicted DIC with high specificity and selectivity. The selected feature genes and model were validated using the *in vitro* rat TG-GATEs dataset.

Discussion and conclusion: This transcriptomic signature has yielded high accuracy in the identification of potential DIC-inducing compounds.

Presentation: Poster

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Update and optimization of an adverse outcome pathway network of chemical-induced cholestasis

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Background and objectives: Cholestasis denotes any situation of impaired bile secretion with concomitant accumulation of bile acids in the liver or in the blood circulation and may be induced by various chemicals. Our group previously introduced an adverse outcome pathway (AOP) network mechanistically describing key events (KEs) and their relationships driving chemical-induced cholestatic liver injury. The aim of the present work was to update and optimize this AOP network in line with guidelines issued by the Organization for Economic Co-operation and Development (OECD).

Material and methods: PubMed was queried for studies of chemical-induced cholestasis using a list of predefined key words and several known KE-related terms. SysRev, a newly developed computational tool for systematic reviewing and data extraction, was employed during the abstract screening and full-text screening. The tailored Bradford-Hill criteria, described by the OECD guidelines, were used in the weight-of-evidence assessment of the KEs and KE relationships.

Results: A total of 6572 articles was retrieved from PubMed and uploaded to SysRev. An initial abstract-screening resulted in a total of 544 papers eligible for data extraction in the full-text screening process.

Discussion and conclusion: Extracted data are used for assessment of already defined KEs and KE relationships, but also for identification of potential new KEs, resulting in an updated AOP network on chemical-induced cholestatic liver injury. The fully assessed AOP network will serve as the conceptual basis for setting up an *in vitro* test battery to identify cholestatic chemicals, consisting of a series of assays that each monitor individual KEs.

Presentation: Poster

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Eye damage reversibility in an *in vitro* model of bovine cornea to replace the Draize test completely

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One of the requirements for chemical product registration such as agrochemicals, is to provide evidence about their potential eye damage. The Draize test performed in rabbits allows the products to be classified into four categories, considering both the severity of the lesions produced in the animal's eye as well as its healing time. The available alternative methods to this live animal test do not allow documenting the damage reversibility, nor the time necessary for such reversibility to occur, as required by the GHS classifications. Our proposal is to complement the *in vitro* model that uses the bovine cornea as a substrate to predict whether a substance is irritating or non-irritating (BCOP), with a strategy that allows predicting if the observed irritation is reversible and the time it takes to revert.

We isolated bovine limbal-stem cells, known to play an important repairing role in corneal injury, and used them as model in a cytotoxicity assay to evaluate the cell sensitivity to reference products. Moreover, a wound healing assay was performed to study whether these products differentially affect the replication and migration capacity of the cells. Furthermore, a tissue explant and an organotypic cornea culture model were implemented to study if the chemical exposure alters cell's replication, migration and overall wound healing differentially.

The results obtained from the cytotoxicity test, the wound healing assay and the explant culture show that these methods are effective to improve prediction to the four categories. Also, damage reversibility was observed in the organotypic cornea culture model.

Presentation: Poster

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Reducing and replacing laboratory animal use in US and UK laboratories

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US and UK Corporations have led the way in developing and implementing alternatives to laboratory animals. While there are good data available on animal use in Great Britain (the animal use data does not include Northern Ireland hence the annual reports do not represent the United Kingdom), in the USA one has to rely mostly on anecdotal reports. However, animal use data does not reflect changes in financial support for laboratory investigations (basic and applied research and toxicity testing). Animal use data and the funding of biomedical investigations in Great Britain will be presented identifying the importance of commercial laboratory efforts in reducing laboratory animal use in Great Britain (and inferring implementation of alternatives) compared to British university laboratories and medical schools.

Presentation: Poster

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Investigating metabolic and reproductive disruption induced by novel flame retardants using an *in vitro* human 3D cell culture model

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Organophosphate flame retardants (OPFRs) have been consistently detected in increasing concentrations in the environmental and human matrices indicating possible human exposure. Therefore, the present study aims to evaluate the effects of OPFRs on human cell culture (monolayer and 3D spheroids) to characterize the toxicological effects and potential mechanisms through morphological, transcriptional, and biochemical assays.

Our findings suggest that the OPFRs, including tricresyl phosphate, triphenyl phosphate, tris(1,3-dichloropropan-2-yl) phosphate, and 2-Ethylhexyl diphenyl phosphate (EHDPP) induced the lipid accumulation in human liver (HepG2) cell culture by altering the expression of genes encoding for hepatic lipogenesis and mitochondrial dysfunction. *In silico* analyses identified PXR and PPARγ as potential molecular initiating events. Moreover,

EHDPP-mediated dysregulation of hepatic lipidome was observed in human HepG2 3D hepatospheroids along with alteration in several genes involved in lipid homeostasis, including ACAT, ABCA1, CYP27A1, GPAT2, PNPLA2, PGC1α, and Nrf2. The human adrenal (H295R) cells exposed to EHDPP showed altered secretion of hormones, including progesterone, androstenedione, and cortisol. Consistently enhanced expression of corticosteroidogenic genes, encoding for cytochrome P450 (CYP11B2, CYP21A1) and hydroxysteroid dehydrogenases (3β-HSD2, 17β-HSD1) was observed. Intracellular lipidomics identified EHDPP-mediated disruption of intracellular lipid profile in 3D hepatospheroids and H295R cells indicated by reduced cholesterol esters, sphingolipids, fatty acyls, and increased phospholipids and triglycerides species, indicating connections between EHDPP-induced metabolic and reproductive pathologies.

In summary, our study identifies several OPFRs as potential risk factors for endocrine-related metabolic and reproductive pathologies that are of increasing importance due to the risk of occupational or cumulative environmental exposure to humans.

Presentation: Poster

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Comparison of *in vitro* oral and topic absorption toxicity of BPA and BPS using 3D cell cultures and microfluidic systems

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Organ-on Chip is an effective solution to pursue new methodologies for drug discovery, toxicity tests and personalized disease treatments. It has been applied as an efficient and predictive solution to beat the high cost of classic toxicity tests using animal models, also reducing the number of animals in experimentation.

Here we propose a junction of three different 3D tissue engineered cultures (skin, intestine, and liver) in a 3-organ-on chip microfluidic device to verify topic and oral Bisphenol A (BPA) and Bisphenol S (BPS) administration. After treatment we evaluate liver toxicity and endocrine disruption. For this, we developed models of human reconstituted skin, intestinal barrier, and liver spheroids, which were characterized in terms of histology, morphology and functionality. Our results show that our model is functional and simulates functions of the real organs. The Chip integration of all the tissues on the chip was well succeeded and improved viability of the 3D cultures. After treatment with Bisphenol A, we observed absorption of drugs, which caused liver injury and genetic modulation of endocrine disruption pathways, as expected. Interestingly, for BPS, which was reported as a substitute for BPA, decreasing toxicity and BPA damage, also induced toxicity and genetic modulation of this markers. In conclusion, here we present a new methodology to screen liver toxicity avoiding animal testes in two contexts, oral and topic administration of drugs. In addition, we found BPS also causes toxicity in liver and also can be a endocrine disruptor.

Presentation: Poster

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A roadmap for regulatory implementation of *in vitro* models for evaluation of vaccine efficacy

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One of the aims of the EU-funded IMI2 project Inno4Vac (Innovations to accelerate vaccine development and manufacture, www.inno4vac.eu) is to create next-generation human *in vitro* 3D models for gastro-intestinal, respiratory and urovaginal mucosae that include relevant immune system components for use in preclinical and clinical studies in vaccine development. These models will be tested and validated using selected pathogens (C. difficile, Norovirus; RSV, influenza; N. gonorrhoeae, HSV-2).

To facilitate the entry of these models into the regulatory arena, we conducted a workshop in 2022 with model developers, industry and regulators to discuss regulatory requirements for these models. From the information gathered, we created a technical roadmap for each model to describe the attributes necessary to succeed in its chosen application.

The roadmap addresses two key questions. First, how does the model add value over existing assays/models for this application? Second, what data need to be collected to be confident the model is relevant for this application? The technical roadmap also serves as a tool to identify necessary standards, assays and comparators and to prioritize work.

The technical roadmap was applied to Inno4Vac models during the Inno4Vac Annual Meeting, held in Siena, along with discussions on model qualification, human variability, characterization and standardization. This process assisted model developers with their next regulatory steps and improved the roadmap. The road-

map will aid in a stage-gate approach to choose the most promising models for further development for regulatory applications.

This work has received support from the IMI2/EU/EFPIA Joint Undertaking Inno4Vac grant no. 101007799.

Presentation: Poster

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A human bone/bone-marrow-on-a-chip approach for *in vitro* culture of human bone marrow and benchmark against clinical reality

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Trabecular microarchitecture of cancellous bone provides not only mechanical stability, but also a microenvironment for reticular connective tissue and vital hematopoiesis in the bone marrow (BM). In joint arthroplasty, one major clinical problem is aseptic peri-implant osteolysis due to local exposure to metallic particles and ions. Previously, we found that the *in vitro* osteogenic capacity of mesenchymal stromal cells (MSCs) is impaired by *in vivo* exposure to cobalt and chromium ions in the BM. For further studies we aim to establish a human bone/BM organ-on-a-chip system to benchmark it to human BM specimens and to validate the system for predicting metal-ion-induced adverse biological effects.

Primary cells (MSCs, osteoblasts and mononuclear cells) from healthy BM were isolated. Human cancellous bone was decellularized, cylindrical scaffolds were prepared, MSCs and osteoblasts were seeded, and matrix mineralization was induced under static conditions. Subsequently, the scaffolds were transferred to an 2-Organ-Chip (TissUse GmbH, Berlin, Germany) and BM mononuclear cells were seeded before osteoclastogenesis was induced by growth factor supplementation. Scaffolds were dynamically cultivated for 21 days without further supplements.

Lactatdehydrogenase quantification revealed cellular integrity over time. Quantification of soluble bone turnover factors indicated active bone metabolism. Fluroscence-microscopy confirmed the presence of active osteoblasts and the formation of reticular fibers. Flow cytometry indicated stable T-cell and monocyte populations as well as detectable levels of B-cells. For toxicity testing, treatment with clinically relevant metal ion concentrations revealed *in vitro* toxicity as seen in patients. This effect could not be observed in a 2D MSC monoculture assay.

Presentation: Poster

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Immune-competent human (multi)organ-on-chips

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One long-term aim of our research group is to create a human immune model system using (multi)organ-on-chip technologies. Due to technological breakthroughs and impressive initial clinical results, innovative immunotherapies represent a focus of current drug developments. Mouse models are poorly suited for testing innovative T cell-based immunotherapies, both cellular and humoral approaches, because, among others, they cannot replicate the "aging" of the human immune system (memory), which has a high impact on the efficacy and safety of the therapeutics. In turn, conventional human 2D immune cell cultures do not adequately represent the important tissue immunity component. Thus, there is a huge need to build a human dynamic test platform for qualifying innovative T cell-based immunotherapies such as CD3 engagers/checkpoint inhibitors and chimeric antigen receptor (CAR) T cells. Our research addresses current scientific challenges such as T cell migration in organoids. For this, we work on vascularization models, test different biomaterials or scaffolds and work on new-designed organ-on-chips. Our group is part of different cooperation projects as the PACE study (vascularization), BCRT NewCrossfields (vascularization), TReAT (new testing model system for ATMPS) and geneTIGA (multi-organ testing model system for ATMPs). Thus, we will work on different organoid models as the liver, intestinal and lung under dynamic conditions.

Presentation: Poster

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Cytokine release from organotypic human oral tissue following exposure to oral care products

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Purpose: Measuring viability of a three-dimensional *in vitro* organotypic human oral tissue model has been suggested as an alternative test method to the hamster pouch oral mucosa irritation test of oral care products. The aim of this study was to investigate the production of two different cytokines using organotypic human oral tissue model following exposure to chemicals that are commonly used in oral care products.

Materials and methods: The organotypic human oral tissues were exposed to ethanol (mouth wash), sodium lauryl sulphate (toothpaste) or hydrogen peroxide (tooth whitening agent) for 90 minutes. Following exposure, interleukin-1α and interleukin-8 productions were assessed and correlated with cell viability testing as well as histology of the organotypic human oral tissues.

Result: High levels of IL-8 were released from organotypic human oral tissues in all of the test and control groups without any significant differences between them. In contrast, differences were found in IL-1α release between the test and control groups. Additionally, the trend of IL-1α release corresponded to the phenotypes observed in histological analysis while different trend existed between IL-1α release and cell viability.

Conclusion: The study concluded the non-specific release of IL-8 for the assessment of oral care product chemicals' toxicity, while potential of measuring IL-1α cytokine level as the possible alternative test method.

Presentation: Poster

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Culture of care – What does it look like at Novo Nordisk

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Culture of Care has gained much attention in recent years after it was introduced in EU Directive 2010/63/EU. Many establishments working with laboratory animals have worked with implementing or developing their own, unique culture and raising awareness when working with animal welfare.

At Novo Nordisk our approach to Culture of Care is what we proactively choose to do when we put legislative requirements into action. We closely look into the intentions of the legal requirements and from that, we choose the solutions which has the best match with the intentions. In other words, – we go above and beyond the minimum requirements of legislation.

Culture of Care also includes the staff working with the animals, as they are the ones providing care to and show empathy with the animals.

Our purpose of working with Culture of Care is to better enable us to reach our goals in relation to the welfare of the animals and of the people working with them.

The presentation will explain how we have worked with mapping the culture of care at Novo Nordisk and how we ensure awareness through the organization on our culture. We will go through examples of Culture of Care in four phases of a study where the laboratory animal veterinarian plays of role, – as a professional and as the person who establishes relationships and collects relevant input from other staff functions. It will also describe our mind-set as well as the supportive elements needed for a healthy Culture of Care.

Presentation: Poster

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From scientific validation to regulatory acceptance in medical devices: Importance of ISO standardization

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Although the 3Rs principles are a central concept for the biocompatibility of medical devices (MDs), many biological tests are still carried out on animals. Integration of validated non animal methods into the International Standardization organization (ISO) 10993 series of standards, is an effective way to accelerate their regulatory acceptance. The prerequisite is to ensure that a method is applicable to the specific context of MD where safety testing is performed on the final MD after packaging and sterilization.

The case of irritation is emblematic of the importance of this standardization work. Although scientifically validated in 2009 for chemicals, and published in 2010 in OECD TG 439, it is not until 2021 that *in vitro* methods started to replace the animal for assessing MD irritation. For skin sensitization, a more complex endpoint, the ISO roadmap is based on a two-step approach. First, the publication of the ISO TS11796 with the requirements for qualification of an OECD method for assessing MD's skin sensitization potential. Then, for qualified methods, integration into the step-

wise approach of the ISO 10993-10 standard for skin sensitization of medical devices.

This presentation will address, through irritation and sensitization of medical devices, the importance of not only scientifically validating non-animal methods but also addressing their applicability to different industrial and regulatory contexts. At ISO, the consensus-based approach to standardization among all stakeholders, including academia, industry, NGOs and regulators, helps build the confidence necessary for the spread and acceptance of these new approaches at all levels.

Presentation: Poster

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Adipocyte spheroids and endothelial rings to evaluate lipogenic or antilipogenic natural ingredients

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A 3D co-culture formed by human adipocytes from adult mesenchymal cells and endothelial cells (HUVECs) were used to assess efficacy of caffeine and plant extracts reporting lipogenic and antilipogenic action. The spheroid model was based on previously established methodology and were set up using 96-well Bio-Assembler™kit consisting of nanoshuttle solution and a plate magnetic drive. The Rings were obtained by magnetic levitation of endothelial cells to assess the *in vitro* vasoactivity of Horse Chestnut, which present a vasoconstrictor effect. The lipogenic or antilipogenic efficacy of the ingredients was evaluated using fluorescent perilipin. Human cells *in vitro* model results showed that the system reproduces the *in vivo* results more faithfully, providing resistance to higher concentrations of the caffeine, tested at 0.375; 0.75 e 1.5 mg.mL-1 concentrations resulting at 208; 87.5 e 48.5% of cell viability respectively; the endothelial cells ring were challenged with Horse chestnut at 0.625; 1,25 e 2.5 mg.mL-1 concentrations and the cell viability were 106; 95 e 86% respectively. Finally, the use of perilipin showed that the spheroids formed are capable of distinguishing between the two concentrations of lipogenic and antilipogenic substances, showing an increase or decrease in the concentration of lipids in the system. The magnetic levitation system used to obtain the spheroids and the rings for the vasoconstriction study are suitable systems for study new cosmetic cellulite treatment or antilipogenic new compounds to be used in anti-aging products.

Presentation: Poster

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Development of fully primary human 3D alveolar model (AlveolAir™)

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Chronic obstructive pulmonary disease (COPD) and lower respiratory infections are leading causes of death worldwide. This highlights the necessity for new and more efficient treatments. In order to develop novel drugs and assess lower respiratory toxicity of xenobiotics, robust and relevant *in vitro* alveolar models would be very helpful. We herein describe the characterization and functionality of a full primary human epithelial-endothelial 3D alveolar model, AlveolAir™.

To characterize AlveolAir™, long-term parameters were measured: Biomarkers for ATIs, ATIIs and tight junctions (CAV-1, HTII-280 and ZO-1 respectively, Immunofluorescence); Morphology and lamellar bodies' presence (Histology & TEM); Existence of a maintained alveolar epithelial barrier (TEER) and SPC secretion (ELISA). Using these techniques, the evolution of the culture was monitored for several weeks.

Functionality of AlveolAir™ was evaluated by exposure to pro-inflammatory compounds (Lipopolysaccharide, TNF-α, polyinosinic:polycytidylic acid (poly(I:C)) and an inflammatory cocktail (cytomix)). TEER and cytotoxicity (Lactate dehydrogenase quantification (LDH)) were monitored daily, along with morphological observations and cytokines quantification (IL-6, IL-8 and RANTES).

Finally, a co-culture model of AlveolAir™ with primary alveolar macrophages has been developed and infected with Streptococcus pneumonia (Sp19F), to assess the activity of macrophages. Compared to AlveolAir™, AlveolAir™-macrophages showed stronger immune response with reduction up to 3.5Log10 CFU after 24 hours of culture.

This novel *in vitro* model, AlveolAir™, represents a relevant and reliable tool for inhalation toxicity assessment of drugs. It is also highly useful for understanding the cellular and molecular mechanisms of respiratory diseases such as COPD, viral and bacterial infections.

Presentation: Poster

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Novel fully primary human airway epithelium-alveolar macrophage *in vitro* co-culture models to study host pathogen interactions

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Being the first line of defense of the organism against airborne pathogens like bacteria and viruses, the respiratory epithelium acts as a physical barrier as well as an efficiency mucociliary escalator. Furthermore, the airway epithelium is also a potent immune-regulator which orchestrates both innate and adaptive immune responses upon bacterial or viral infections.

Many animal models have been used to study lung infections, but the relevance and predictability of animal models are still questionable. Here we established a new co-culture model using well characterized, standardized human airway epithelium such as MucilAir™, SmallAir™ and human lung macrophages (CD45+,HLA-DR+, CD206+, CD11b+and CD14-) for studying bacterial and viral infections. The alveolar macrophages were not only able to adhere to the epithelial cells, but also functional: The macrophages were capable of phagocytosis, evaluated using pHrodo™ Red (S cerevisiae Bio-particles Conjugate). Moreover, the co-culture models respond to pro-inflammatory stimuli such as LPS, TNF-α and Poly(I:C) with an increased IL-8 secretion.

Upon bacterial infection with methicillin-susceptible *Staphylococcus aureus* strain (MSSA), compared to MucilAir™ monocultures, MucilAir™-macrophages showed stronger immune responses: (i) a reduction of bacterial growth (up to 1.5Log10 CFU) and (ii) decreased upregulation of IL-8 and β-defensin-2 secretions. Interestingly, greater difference was observed for Streptococcus pneumonia (Sp19F): The presence of macrophages led to a decrease of 3.5Log10 CFU after 24 hours of culture (N = 12) versus MucilAir™ alone.

These novel *in vitro* models might find applications in understanding the role of immune-epithelial cell interactions in infection diseases and inhalation toxicity assessment.

Presentation: Poster

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An updated adverse outcome pathway network for chemical-induced liver steatosis

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Background and objectives: Adverse outcome pathways (AOP) are frameworks depicting existing information on causal linkages (i.e., key event relationships (KER)) between measurable biological changes (i.e., key events (KE)) leading to an adverse outcome. To better represent complex interactions within organisms, different AOPs sharing one or more KEs are brought together in an "AOP network". The aim of this research was to update the current AOP network on chemical-induced liver steatosis. Furthermore, to weigh the evidence between KEs, the updated AOP network was also assessed in accordance with the specific guidelines from the Organization for Economic Co-operation and Development (OECD).

Material and methods: PubMed was used to collect publications on chemical-induced liver steatosis using a list of predefined steatosis search terms and KE-associated keywords. A title/abstract screening and full-text screening of collected papers was performed with SysRev (i.e., a computational tool for systematic reviewing and data extraction) using two labelling strategies. Subsequently, extracted data was used to assess the level of confidence in the updated AOP network on liver steatosis according to the tailored Bradford-Hill Criteria.

Results: The PubMed search resulted in 12,478 papers. The title/abstract screening resulted in 1,626 papers eligible for data extraction in the full-text screening phase.

Extracted data was used to assess the level of confidence in previous described KEs and KER. In addition, data was used to identify potential novel KEs.

Discussion and conclusion: The updated AOP network on liver steatosis will serve as a basis for the development of animal-free methods for toxicity testing purposes.

Presentation: Poster

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Expanding the applicability domain of NAMs for skin sensitization testing: A case study using GARDskin for assessment of metals

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New Approach Methods (NAMs) for detection of sensitization have been validated and adopted as OECD TGs during the last decade. These assays target different Key Events (KE) in the AOP for skin sensitization and are increasingly being applied to replace animal models within different chemical sectors. However, further characterization of the applicability domain (AD) of these assays is critical to understand limitations and to facilitate regulatory uptake in other industrial sectors.

Of particular interest from a scientific and regulatory perspective is the potential to use NAMs for assessment of metals, which have been proposed to act via alternative mechanisms to organic chemicals. The current study describes a joint effort by industry and assay developers to evaluate the AD of the GARDskin assay for metal compounds. GARDskin is the first harmonised method utilizing a combination of genomics and machine learning for a regulatory endpoint and was recently adopted into OECD TG 442E.

A selection of metal salts (n = 13) was evaluated and the accuracy, sensitivity, and specificity for prediction of skin sensitizing hazard of metals were estimated to 92% (12/13), 100% (7/7) and 83% (5/6), respectively. Interestingly, transcriptomic analysis revealed almost identical response patterns in dendritic cells for metals and organic compounds, indicating a high similarity in the toxicity pathways driving classifications.

In conclusion, the result from this study supports inclusion of metals into the AD of GARDskin, which is an important step to ensure scientific/regulatory confidence to reduce the need for animal testing within the metal production and medical device sector.

Presentation: Poster

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MiceAId: Using the AI to help identification of pup's gender

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Lab animal facilities need efficient control of animal production in order to avoid wasting lives, supplies and related costs. The zootechnical disposal of neonates up to 5 days old in breeding lab animal facilities is a management alternative that also enables the most ethical humanitarian endpoint in animals that will not be used by demand, being the accurated sex identification of neonates crucial, carrying out the disposal only of animals with characteristics not requested by the researchers, highlighting, for example, the majority use of male animals. However, newborn sexing is a technique that has not been widely correctly applied, requiring training and constant practice for accurate use. The Innovation Lab of ICTB/Fiocruz and CAMBE-UFRJ developed the MiceAId app that allows the sexing of newborns of mice from 3 different strains (Swiss, BalbC-An and C57/BL6) with the application of deep learning techniques (neural network convolucional-CNN) through the use of computational vision in YOLO framework for classifying characteristics of the genitalia of newborns. The application can be used by a mobile device (tablet/cell phone) and during the Proof of Concept presented 96% accuracy in identifying males in the Swiss and BalbC-An strains. Iterations are being applied to learn the female gender identification algorithms and improve the prediction rate (currently around 60%). The use of the app can help in the management of the lab animals used in research, reducing the rate of errors in sexing and increasing the precision in zootechnical disposal.

Presentation: Poster

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Human eythropoietin pharmaceutical product potency: An *in vitro* analytical method implementation

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Human Erythropoietin (EPO) acts on erythrocyte predecessor cells by stimulating their proliferation, differentiation, maturation and inhibiting apoptosis, thus increasing erythrocyte production. EPO is clinically used for the treatment of anemia associated with chronic kidney failure, cancer, HIV infection, pre and post-operative, rheumatoid arthritis and bone marrow transplantation. The potency test (biological activity) of EPO is still described today, in official compendiums, as an *in vivo* test. However, with the growing ethical care in the use of animals in experiments and the variability of results, it becomes interesting an *in vitro* alternative method that directly impacts ethical, financial and analytical issues, resulting in safety and quality to potency tests. To address these questions, the development of an *in vitro* analytical method for application in the pharmaceutical industry consists of evaluating the proliferation of the TF-1 cell line according to the stimulus received from EPO, having as reference a primary pharmacopeial standard. The results showed that TF-1 is capable of responding to the EPO stimulus, showing dose-response curves between 0.15 IU and 5.00 IU with linear cell growth and r2 = 0.99. The implementation of this method will considerably reduce the use of animals in the industry, assessing an appropriate quality control of the product potency as well as leads to a transition toward animal-free testing and more human-relevant results.

Presentation: Poster

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Inhalation toxicity mechanisms of legacy and next-generation perfluoroalkyl and polyfluoroalkyl substances (PFAS)

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Per- and polyfluoroalkyl substances (PFAS) are complex synthetic compounds used in several products and industrial processes. PFAS are mobile, omnipresent in the environment and extremely persistent. Although the toxicological implications of human exposure to certain (legacy) PFAS are well-studied, data on alternative/next generation PFAS are still very limited. Thus, collecting experimental toxicity information of such poor-data PFAS is of high importance.

The high serum-binding of PFAS congeners may influence their cytotoxic activity. Therefore, the influence of foetal bovine serum (FBS) and bovine serum albumin (BSA) on the cytotoxic activity of one legacy (perfluorooctanesulfonic acid – PFOS) and seven next-generation PFAS belonging to the sulphonic acid series was investigated. A simple *in vitro* alveolar epithelial barrier model, i.e. A549 cells was exposed to the compounds in the presence of zero, low (1%) and high (10%) FBS, or BSA (1 mg/mL). Cells exposed to PFOS in the presence of BSA displayed an increased susceptibility to cytotoxicity than cells exposed in the presence of any or no level of FBS. Although this was observed for most of the poor ¬data PFAS, some compound specific differences were observed.

Future studies encompass the use of an advanced *in vitro* 3D co-culture model of the alveolar barrier coupled with single-cell transcriptomics. The results will inform on potential apical effects that can be measured in high throughput assays, and so contributing to a testing toolbox to improve and address the hazard of PFAS exposure to human health and ultimately, facilitate read-across actions between legacy and poor-data congeners.

Presentation: Poster

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Microfluidic model of innervated human skin

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In vitro models of human skin are availableå for many years now and they are being widely used to access skin irritation, corrosion, UV exposure, etc. However, existing models lack tactility and sensitivity. Due to the difficulty in obtaining human neurons, several groups have been using non-human neurons in order to enrich functionality of existing *in vitro* models.

Here, we report the use of functional hiPSCs (human induced pluripotent cells) derived peripheral sensory neurons to establish a co-culture *in vitro* model with primary human epidermal keratinocytes. The co-culture was established employing a microfluidic technology (Ananda Devices, Canada) specially designed to evaluate potential functional interactions between neurons and other cell types. The microfluidic device improved axonal growth, neuronal subtype homogeneity and maturity of the innervating sensory neurons.

This proposed "neuroskin-on-a-chip" *in vitro* model can be potentially used as alternative methods testing platform to study signal transduction in the screening of biomolecules acting on both keratinocytes and sensory neurons cross-talk.

Summary: In this study, we constructed a miniaturized model of innervated human epidermis in a microfluidic platform consisting of human keratinocytes and hiPSCs derived sensory neurons, and potentially useful in the cosmetic and pharmaceutical industries.

Presentation: Poster

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Testing short-chain fatty acid effects on the efficacy of CAR T cells in a gut-on-chip system

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Pre-treatment of cancer patients prior to CAR T cell therapy significantly alters the level of short-chain fatty acids (SCFA) in the intestine, thereby potentially influencing the efficacy and the safety of adoptive cellular therapy (ACT) with CAR T -cells.

To investigate the underlying effects of SCFAs, we used an immunocompetent microphysiological system of the gut expressing specific target proteins of therapeutic CAR T cells. Our studies could confirm the specific anti-tumorigenic activity of T cells in the intestinal tumour model, which was reflected by severe tissue damage. However, pre-treatment of CAR T cells with SCFAs limited tissue damage in a dose-dependent manner, depending on the type of the applied SCFA, with butyrate mediating the strongest CAR T cell inhibitory effect. Tumour protective effects were recapitulated by increased apical junction expression, the ameliorated release of proinflammatory cytokines release and increased cell viability. These effects, however, were abrogated upon inhibition of histone deacetylases (HDAC).

We were able to show that SCFAs modulate anti-tumorigenic CAR T cell activity in a dose-dependent SCFA type-dependent manner in an immunocompetent gut-on-chip model via modulation of the HDAC pathway. In future studies, we will establish an isogenic intestinal model based on human induced pluripotent stem cells to study the modulation of CAR T cell effects by SCFAs in a patient-specific context to identify personalized biomarkers for a more effective and safer ACT for cancer treatment.

Presentation: Poster

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Transgenic zebrafish larvae as a non-rodent alternative model to assess neutrophil responses to nanomaterials

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The exploitation of nanomaterials (NMs) in diverse products (e.g., food, cosmetics, textiles, medicines, electronics) is increasing. Assessment of NM hazard is challenging given the rapid growth of nanotechnology and the production of a huge diversity of NMs. NM hazard assessments commonly focus on whether NMs activate an inflammatory response, with a reliance placed on rodents. Zebrafish (*Danio rerio*) are not protected until they have reached the stage of exogenous feeding at 5 days post-fertilisation (dpf) and we propose that they are used as an alternative to rodents in nanotoxicology to enhance implementation of the 3Rs principles. The aim of this study was to investigate the suitability of using transgenic zebrafish as a test model for screening NM toxicity via assessment of neutrophil responses. Transgenic zebrafish (Tg(mpx:GFP)i114) with fluorescently-labelled neutrophils were exposed to silver (Ag) and zinc oxide (ZnO) NMs at 3dpf via water (following a tail fin injury), or via microinjection into the otic vesicle and neutrophil accumulation at the injury or injection site quantified at 4-48 h using fluorescent microscopy. Both NMs activated an enhanced neutrophilic inflammatory response in injured zebrafish following aqueous exposure. Ag NMs stimulated the greatest inflammatory response. Ag NMs also stimulated neutrophil accumulation in the otic vesicle, which peaked at 48 h. We suggest that transgenic zebrafish can be effectively harnessed to rapidly screen NM toxicity via assessment of inflammation and provide us with considerable potential for reducing our reliance on rodent studies in nanotoxicology (and other disciplines) to make testing more ethical, quicker, cheaper and predictive.

Presentation: Poster

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Multiclonals™ and beyond: The power of animal-free antibodies

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Today, most antibodies for research and diagnostics are still produced in animals. Although the use of animal-free antibodies in therapeutic applications has been very successful, the research and diagnostic field are still lacking a profound range of antibodies from animal-free sources.

With the "Recommendation on non-animal-derived antibodies" of the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) from 2020 a new era of perception has begun. However, an estimated one million animals are still used annually for antibody production and development within the EU.

We provide a completely animal-free solution to replace all current types of products on the research and diagnostics antibody market:

1. Monoclonals (hybridoma-derived antibodies)
2. Recombinant antibodies which are derived from immunizations
3. Serum products (polyclonals)

Our vegan antibodies are made completely *in vitro* without immunization by antibody phage display. Additionally, every single animal-derived material in the entire process has been replaced, e.g., in cultivation media or blocking reagents, resulting in truly vegan antibodies.

Multiclonals™, our polyclonal replacement, have three advantages for your work in addition to being vegan. First, they are truly target specific, eliminating the background reactivities of serum products. Second, they have unlimited reproducibility due to their recombinant production in cell culture. Third, they are customizable, e.g., by modifying an Fc part suiting your needs.

Consequently, vegan antibodies can even offer improved versatility compared to animal antibodies. Additionally, they save time, costs and meet future ethical requirements in research and diagnostics as a higher quality and sustainable substitute to animal-derived products.

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Sharp increases in US animal research post-COVID

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Metrics tracking research animal use in the United States are extremely limited, obscuring the ability to measure the impact of reduction and replacement efforts over time. Normally, the U.S. Department of Agriculture (USDA) has reported national summaries of animal numbers from registered research facilities via annual reports required under the federal Animal Welfare Act (AWA), but the agency has been inconsistent in maintaining these records online. In the interest of making the summaries available to the public, we compiled animal numbers from 2014 to 2021 as reported to the USDA. We found that, despite a slowly decreasing trend in usage of animals from 2014 to 2019, there was a sharp increase in all AWA-covered species excepting cats and pigs during 2020 and 2021. Since the COVID-19 pandemic began, approximately an additional 35,000 hamsters, 25,000 rabbits, 12,000 nonhuman primates, 9,200 guinea pigs, 4,700 dogs, and 1,700 sheep were reported as used by USDA-registered facilities, resulting in a total increase of 16% from 2019 to 2021 of AWA-covered animals used each year. It is important to note that that upwards of 99% of all animals used in U.S. research laboratories are not covered by the AWA, including most mice, most rats, and all cold-blooded animals. Still, the increase in the use of AWA-covered animals reflects a pronounced disconnect between public health needs and our current research investment. In addition, this demonstrates the need for greater government transparency around the numbers of animals used in research each year in the US.

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Human-on-a-chip: History, progress, promise

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Human microphysiological or "Body-on-a-Chip" systems are powerful tools to assess the potential efficacy and toxicity of drugs in pre-clinical studies. Having a human based, multiorgan system, that emulates key aspects of human physiology can provide important insights in the decision about which drugs to move into clinical trials. I will briefly describe the history and concepts behind this technology and then recent applications. Human surrogates are constructed using a low cost, robust "pumpless" platform. We use this platform in conjunction with "functional" measurements of electrical and mechanical activity of tissue constructs (in collaboration with J. Hickman, University of Central Florida). Also, by combining PBPK-PD models with these devices we can enhance our predictive power for anticipating human responses. Using a system with four or more organs we can predict the exchange of metabolites between organ compartments in response to various drugs and dose levels. We have constructed models incorporating barrier tissues such as GI tract, blood brain barrier, and skin with internal organs such as liver, cardiac, and neuromuscular junctions and an immune system mimic. With these systems, we can predict both efficacy and toxicity of drugs in humans from preclinical studies. This system provided all the efficacy data necessary to move a drug candidate for rare neuromuscular diseases into a Phase Clinical trial (by Roche) We believe that these "Body-on-a-Chip" systems have great potential to increase the efficiency of conversion of drug candidates into approved, successful pharmaceuticals.

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Mapping a new paradigm for agrochemical carcinogenicity assessment

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The rodent cancer bioassay is currently required by most regulatory authorities for assessing human carcinogenic potential for agrochemicals, food additives, industrial chemicals, and pharmaceuticals. The length of time to perform the bioassay and the limited ability to address human carcinogenicity has led to many efforts to modernize carcinogenicity assessment away from the lifetime rodent cancer bioassays. For example, weight of evidence-based assessment of *in silico*, *in vitro*, and short-term *in vivo* tests have the potential to substantially reduce animal use while still protecting public health and better determining human carcinogenic hazard and risk. As a result, multiple international initiatives are developing frameworks for regulatory decision-making that will ultimately enable the replacement of the rodent cancer bioassays.

In this context, leaders of the Rethinking chronic toxicity and carcinogenicity assessment for agrochemicals project (ReCAAP) and the European Partnership for Alternative Approaches to Animal Testing (EPAA) workgroups came together and recognized the need to map international efforts to develop and adopt new approaches for carcinogenicity assessment. Several ongoing initiatives were identified, including (but not limited to) ICH S1, EPAA, OECD IATA for chemical non-genotoxic carcinogens, ReCAAP, and the NTP Health Effects Innovation. Given the overlapping objectives of these initiatives to reduce use of the rodent cancer bioassay, the authors scoped a landscape that identifies opportunities for collaboration (bringing together international experts from governments, industry, academic, and non-government organizations) to build a roadmap that leads to global acceptance and incorporation of fit-for-purpose new approaches for human-relevant carcinogenicity assessment of agrochemicals.

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Engineering metabolically active reconstructed human skin for organ-on-chip

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Background: Considering the skins major barrier function, it is expected that it would be a metabolically very active organ. However, current reconstructed human skin (RhS) models do not adequately reproduce the metabolic potential of native human skin.

Aim: To determine whether the incorporation of an adipocyte containing hypodermis into RhS will improve its metabolic potential and to determine which major metabolic pathways are involved.

Methods: Primary human keratinocytes, fibroblasts and differentiated adipose-derived stromal cells (ASCs) were co-cultured to create an adipose-RhS (reconstructed epidermis on fibroblast populated hydrogel integrated above adipose layer).

Results: Lipid droplet formation, gene expression of key adipogenic markers and adipokine secretion confirmed successful differentiation of ASCs to adipocytes. Epidermal integrity was maintained. Addition of the adipose layer resulted in down-regulation of 9 and up-regulation of 286 genes in the dermal-adipose compartment compared to RhS with only the dermal compartment. Out of the up-regulated genes, 5 were identified as phase I, and 2 as phase II metabolic enzymes. Gene ontology analysis revealed that these were mostly involved in vitamin A and vitamin D metabolic pathways. The cytokine secretion profile changed drastically, showing reduced concentrations of pro-inflammatory cytokines in adipose-RhS compared to RhS.

Conclusion: Adipose-RhS has a less inflamed phenotype indicating the contribution of adipocytes to tissue homeostasis. Up-regulated phase I and phase II enzymes shows higher metabolic activity of adipose-RhS compared to RhS. Therefore, adipose-RhS mimics native skin more than traditional RhS and hence is a better model for investigating human skin in health and disease in Organ-on-chip

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Introducing the 3Rs concept in higher education: Experiences in the Universitat de Barcelona

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The 3Rs concept (Replacement, Reduction and Refinement) is a globally recognized framework for ethical use of animals in scientific experimentation. In an educational setting, the 3Rs can be taught to student as part of their science curriculum to raise awareness about the ethical considerations involved in animal experimentation and encourage the use of alternative methods. However, there are limitations to introduce this in curriculum. The first is related to the organization of the curricula and the difficult to introduce changes in them properly, which require the national administration of higher education authorization. The second is that although researchers and professors are more familiarized with reduction and refinement, their knowledge on replacement is limited or insufficient. Then the most important is to introduce students but also professors in the new methodologies related to replacement. In that case, to solve these limitations we can follow different strategies: a) guest lectures and workshops for students and professors inviting experts in alternative methods; b) providing opportunities for hands-on experience offering students the opportunity to participate in research projects that use alternatives; c) encouraging students to apply the 3Rs in laboratory practicals, etc. Following such recommendations and strategies, we present here our activities in the faculty of Pharmacy and Food Sciences related to final degree projects, seminars, short courses and involvement of students in our research projects related to the development of *in vitro* methods to replace animals.

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Environmental thermal refinement and its impact on energy efficiency and cholesterol in mice

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Mice experience various chronic stressors in the laboratory environment, such bright lights and cold stress. Chronic stress is well known to affect many aspects of physiology but in the case of mice, these stressors may alter energy expenditure, weight gain, and overall lipid profile; confounding the ability to model changes in cholesterol and overall metabolism in laboratory mice. Therefore, we hypothesized that refining the mouse's environment, through dark cages (red tint) and thermal access, would alter aspects of metabolism. A factorial design was utilized to assess the following treatments: Temperature (no heat-20C; 1/3 of the cage floor heated to 30C) and cage color (red caging to reduce light intensity; clear caging = typical lighting) in male and female C57BL/6J (N = 32 cages; 2 mice/cage = 64 mice; 8 weeks old). Mice and their food hoppers were weighted weekly. At the end of 4 weeks, mice were euthanized to evaluate cholesterol (total; HDL; LDL). Data were analyzed as General Linear Models in JMP. Mice in clear cages with no heat (20C), had the highest feed:gain ratio compared to all other cage combinations, indicating poorer energy efficiency ($F_{1,1} = 7.24$; $P = 0.13$). However, total circulating cholesterol, HDL, and LDL levels were unaffected by environmental treatments. It appears that environmental refinements that are more tailored to the animal's specific needs, including lighting, can affect energy efficiency but does not appear to alter circulating cholesterol levels in mice.

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Training assessors on establishing competency of staff using animals for scientific purposes

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Animals used for scientific purposes must be treated humanely, which warrants responsible care and use by competent staff. Competence can be broadly defined as performing an activity at a high standard, combining competency in performing the required tasks with desirable personal attributes. It is achieved through education, training, experience, and continuing professional development, and must be regularly assessed.

To ensure animals are used and cared for competently, principles and practices must be assessed reliably, objectively and transparently. However, establishing trust in competency to perform procedures autonomously is challenging, as it needs certifying that those being assessed not only hold the necessary knowledge and mastery of practical skills, but also that they manifest appropriate attitudes of empathy towards animals and act accordingly, the latter requiring assessing both trainees and established professionals in their daily work. Here, a five-step approach for assessing competency in a specific task is proposed, reducing the supervision required with each step, until one can be entrusted to fully work independently.

Working for the European Commission, we have defined harmonized learning outcomes for training of competence assessors, based on which we are developing online training resources. Contents will cover the value of training and assessment; developing and performing assessments; enhancing and establishing trust within a performance; and reflecting on the assessment process. This is inspired by medical education, where "entrustable professional activities" are becoming key components of establishing competency in patient care, and which can now be applied to establish competency in animals used for scientific purposes.

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An improved ALI exposure chamber for higher deposition efficiency and optimized operations

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In respiratory *in vitro* toxicology, air-liquid interface (ALI) exposure chambers are a promising method for cell culture exposure. However, many ALI exposure systems are characterized by several limitations (e.g. low deposition efficiency – 35-60% for single-droplet deposition systems, risk of chemical carryover, time-consuming operations). The ALI system, on the contrary, should be able to mimic *in vivo* conditions (inhalation) while ensuring high and controlled deposition efficiency, especially when used to deliver valuable compounds (e.g., environmental particles, pharmaceuticals APIs).

To overcome these issues, we developed an ALI exposure system with both housing and casing equipped with an integrated heating mean, thus enabling a non-intrusive heating and temperature monitoring within the housing, and quick temperature rising in the exposure chamber. This innovation allows much higher deposition rates, thanks to the reduced condensation of the aerosol on the casing walls. Additionally, we modified the lower chamber to allow housing of different format of multiwell plates. A disposable pierced lid keeps the hanging wells in place while significantly reducing the risk of chemical carryover and biological contamination. Furthermore, the time needed to set-up and clean the system post-exposure is significantly reduced.

To assess the deposition efficiency, sodium fluorescein was aerosolised and quantified after deposition on Transwells. We found out that the deposition was 4-5 times higher in the double heated system compared to the conventional single-droplet deposition system. The use of the disposable lid reduced the operation time by 80%, removing the risk for chemical carryover and biological contamination linked to the exposure operations.

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Ensuring the quality of a test system using the principles of Good *In Vitro* Method Practices (GIVIMP): A case study of cryopreserved human precision-cut lung slices

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Human precision-cut lung slices (hPCLS) are a highly relevant 3-dimensional model of the lung. They offer native architecture and cells of the lung tissue including respiratory parenchyma, small airways, and immune competent cells involved in inflammatory and sensitization processes. The scarcity of human lung tissue available for research and the inability to conduct larger scale testing has limited the use of hPCLS as a test system for routine, high-throughput testing.

To overcome this barrier, IIVS has refined the methodology behind the cryopreservation, storage, thaw and post-thaw maintenance of human lung slices. As primary tissues have varied quality and responsiveness, a standardized performance characterization (PC) is conducted on all donor batches. To establish credibility for the approach, the principles of the OECD guidance document (No. 286), "Good *In Vitro* Method Practices (GIVIMP)", were applied. Documentation for 1) Donor batches created (including blinded donor demographic/medical data), 2) hPCLS physical and functional characteristics (e.g., PC data) and storage conditions, 3) recommended thaw and culture protocol, and 4) chain of custody documents relevant to the distribution of hPCLS to other laboratories are maintained.

The quality principles of GIVIMP are crucial to the hPCLS serving as a reliable test system for use in repeatable research and regulatory toxicology. With improvements in slice creation, storage, culture conditions, and the quality framework surrounding all of these efforts, the IIVS hPCLS can be confidently used for larger scale testing, tissue banking, and repeat donor experimentation while retaining tissue integrity and functionality, both in a research and regulatory context.

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Removing the mouse from the house: An ELISA method to replace mouse-based potency testing for pertactin antigen

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There is increasing interest to replace animal-based potency assays used routinely to test vaccines, since they are highly variable, costly, and present ethical concerns. The development of relevant *in vitro* assays is part of the solution. Using the pertactin (PRN) antigen as an example in DTaP-IPV (diphtheria, tetanus, acellular pertussis, and inactivated poliovirus) vaccines, a PRN antigenicity ELISA was developed using two monoclonal antibodies with: 1) high affinity to unique PRN epitopes, 2) relevance to human immune responses, and 3) evidence of functionality. The ELISA measured consistent PRN antigenicity between vaccine lots, and was validated to demonstrate its accuracy, precision, linearity, and specificity. Notably, the PRN antigenicity ELISA was more sensitive than the mouse-based potency test and could more effectively differentiate between degraded and intact vaccine lots, compared to the *in vivo* test. From these studies, the PRN antigenicity ELISA is proposed as an *in vitro* replacement for the *in vivo* potency test for PRN in DTaP-IPV based formulations. Important considerations in this study included comprehensive antibody characterization, testing of multiple vaccine lots, method validation, and comparison to animal-based potency. Together, these factors form part of an overall strategy that ensures reliable and relevant *in vitro* assays are developed to replace animal tests.

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Jason Szeto is a Sanofi employee and may hold shares and/or stock options in the company.

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A working group supporting adoption of MPS in infectious disease research

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As animals and humans share some anatomical and physiological similarities, animal studies have contributed to a foundational understanding of the biological processes involved in disease. However, usefulness of animal models in understanding human health is limited, partly due to inherent species differences. Scientific and technological advances that attempt to address these limitations include the development of *in vitro* platforms called microphysiological systems (MPS). Thus, human cell-based MPS have demonstrated their potential to unravel mechanisms of human physiology and disease by recapitulating human organs and organ systems in a dish.

The emergence and global spread of COVID-19 presented an opportunity for assessing the utility of MPS to study how SARS-CoV-2 affects the lungs and other organs and to support development of therapeutics. However, these rapid and widespread endeavors also increased the risk of overlapping investigations and duplication of research efforts. The MPS for COVID Research (MPSCoRe) working group was organized to globally connect key MPS stakeholders to reduce this risk. The working group facilitates open communication among stakeholders to maximize the impact of MPS technologies in understanding disease mechanisms and treatments and reducing animal use while improving human health. In this way, the group aims to promote adoption of MPS for studying COVID-19 and future emerging infectious diseases. These efforts will accelerate the development and adoption of MPS in infectious disease research and may reduce the reliance on animal models in future studies.

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Impedance based prediction of eye irritation

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Ocular irritancy testing on animals for safety reasons such as the Draize eye test are still prevalent around the globe. In the Global Harmonized System (GHS), neat chemicals are categorised and labelled along their eye irritation severity into category 1 for severe eye damage and category 2 for eye irritation. The Draize test only provides neat-related substance testing to reduce animal suffering. However, consumer goods are often based on diluted substances. Therefore, a method to identify concentration dependent eye irritation potential of a chemical is of great interest for risk assessment.

To address this challenge, we examined effects of concentration-dependent eye irritation employing a modified protocol of the Organisation for Economic Co-operation and Development (OECD) 492 test guideline. Instead of an MTT-assay, we used non-destructive impedance spectroscopy to analyse reconstructed cornea like models based on primary human cells. We tested four category 1 substances in three different dilutions: 100%, 5% and 1%. Tracking concentration dependent damage, we measured impedance 6 times over 11 days. The transendothelial or transepithelial electrical resistance at the frequency of 1000 Hz decreased to below 10% in undiluted category 1 chemicals, indicating severe eye damage. However, with the dilution the TEER values increased above 80% of the control indicating a non-irritant and a dose dependent effect. These findings were supported by morphologic analysis of H&E staining in the tissues, displaying dose dependent damage.

Impedance-based predictions could influence the labelling of formulated consumer goods and pave the way to alternative tests.

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A novel multiplex immunoassay for animal-free quality control of DTaP vaccines

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Routine batch quality testing before vaccine release, notably for potency determination, still relies on animal use for several veterinary and human vaccines. Our study is part of the VAC2VAC project, a public-private consortium of 23 partners funded by EU, whose main objective is to reduce the number of animals used for batch testing by developing immunoassays that could be implemented for routine potency assessment of vaccines. The research presented here focused on the development of a Luminex®-based multiplex assay to monitor the consistency of antigen quantity and quality throughout the production process of DTaP vaccines (Diphtheria, Tetanus, acellular Pertussis) from two human vaccine manufacturers. In-depth characterized monoclonal antibody pairs were used for the development and optimization of the Luminex® assay with non-adsorbed and adsorbed antigens as well as with complete vaccine formulations from both manufacturers. The multiplex assay demonstrated good specificity, reproducibility and absence of cross-reactivity. Analysis of over- and under-dosed formulations and heat or H_2O_2-degraded products revealed the ability of the assay to detect qualitative and quantitative alterations of formulated antigens. Finally, the evaluation of 11 primer vaccines from manufacturer A, 29 primer and 28 booster vaccines from manufacturer B demonstrated an acceptable batch to batch variability (CI: 82-122%) when compared to a reference homologous batch, except for the pertussis toxoid from manufacturer B. The results generated in this study provided the proof of concept for a future application of the multiplex immunoassay as a useful tool in the frame of DTaP vaccine quality control.

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The 3Hs – A holistic approach to refinement

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Rather than focussing on refining an individual procedure, we have adopted an approach that looks at the entire lifespan of the laboratory animal and seeks to improve their experience by targeting the 3Hs: Housing, Habituation and Handling.

Our animals are housed in highly enriched cages to promote natural behaviours and reduce stereotypical behaviours associated with stress. We also use playpens for added enrichment and have demonstrated the positive impact that they have on welfare.

We have implemented a habituation protocol which includes basic handling, modified restraint methods and exposure to experimental apparatus to develop positive associations before the beginning of the experimental procedure. This can decrease the stress of the animal across all procedures as the animal has a positive association with the handler.

We have modified our restraint techniques to eliminate the need for "scruffing" in rats or tail handling in mice for substance administration. We have used objective measures of affective state and the stress response to show that these methods offer significant improvements in welfare. We have also developed an oral dosing protocol that eliminates the need for gavage dosing as the test substance is combined with a palatable substance that the animal drinks directly from the syringe.

By taking a holistic approach and addressing the animal's relationship to both its environment and research staff we can reduce the cumulative stress across the entire lifetime of the animal.

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Animal Cranio Kit: A simulator to replace rats in craniotomy training

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Rats are animals commonly used in neuroscience research because they are easier to handle and manage. The current and future scenario denote an increase of neurodegenerative diseases cases, but our understanding about the origin and control of these diseases

is still limited. For this reason, laboratory animals are still widely used in research and in some practices in biomedical sciences courses, where the practice of craniotomy stands out, an operative technique that enables access to the brain.

The Animal Cranio Kit is a rat craniotomy simulator developed to replace them in training and to refine the experimental technique after training. The simulator helps the training of scientists in early stage who have neuroscience projects performing animal experimentation, but never had any kind of training in invasive operative techniques such as craniotomy, which leads to unnecessary deaths of many animals due to malpractice during the experiment.

The simulator was developed in 3D printing with thickness and resistance of the head similar to the structure of the rat skull and contains material that imitates the dura mater and the brain, providing a more realistic experience in craniotomy training as many times as the user intends to use it, until feel safe to perform the procedure on the animals.

The Animal Cranio Kit is currently in TRL 7, having received a positive evaluation from the test users. The simulator has a patent required in Brazil and in several countries (INPI 923521771/ WO 2021/146786 A1).

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Extended-release buprenorphine in a murine model of acute kidney injury

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We wanted to test whether an extended-release BUP formulation offers an efficacious and safe post-surgery analgesia in a mouse model of ischemia/reperfusion-induced acute kidney injury (AKI) without influencing renal parameters.

First, we assessed in healthy C57BL/6 mice, blood and brain levels of a microparticulate extended-release BUP formulation [1] vs. Temgesic® and monitored activity levels in digital cages (DVC®). Then, we compared in an acute kidney injury model over 24 h post-surgery the extended-release BUP formulation (single s.c. injection) vs. Temgesic® (3 s.c. injections followed by overnight application in drinking water) and measured BUP effects on renal function parameters, clinical condition, and Mouse Grimace Scale as non-invasive examination of distress and pain in mice.

Our results in naïve mice demonstrate that the extended-release BUP formulation kept brain buprenorphine levels above 1 ng/ml for > 24 h, while they quickly fell below detection limit after Temgesic® injection. Activity of naïve mice was slightly increased by the extended-release BUP formulation. Importantly, the extended-release BUP formulation did not affect renal function and body weight of AKI mice, while the clinical scores of general well-being were slightly improved esp. during the first 12 hours post-surgery. The Mouse Grimace Scales demonstrated non-inferiority of the extended-release BUP formulation.

Our results 1) confirm that the microparticulate extended-release buprenorphine is a safe and efficacious analgesia treatment in mice with renal impairment and 2) validate the Mouse Grimace Scale as non-invasive monitoring option of pain amenable to automated analysis by deep learning.

Reference

[1] Schreiner et al. (2020). *Sci Rep.*

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Be brave to advance alternatives: A trade association's role in NAMs promotion and education

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Trade associations are perfectly positioned to promote acceptance and use of NAMs through their trusted relationships with industry, regulators and NGOs.

With the UK establishing new chemicals regulatory strategies there is a unique opportunity to integrate NAMs, from the beginning, into frameworks considering exposure along with hazard, to understand real-life human or environmental risk.

In March 2022, CTPA, the UK cosmetics trade association, convened an expert NAMs workshop, connecting industry scientists, testing houses, NGOs, academia and Government to:

- bring together isolated activities, all working towards a common goal;
- champion outcomes-focussed approaches to chemicals legislation, avoiding prescriptive methods;
- identify NAMs best practice and integration into chemicals safety assessment alongside traditional data to progress regulatory acceptance and promote wider use.

However, regulatory acceptance cannot inspire change unless paired with education. As a sequel to the workshop, in 2023 CTPA convened industry experts to deliver practical, case-study centred training in animal-free human health and environmental assessments. Industry and regulatory scientists gained confidence in using NAMs in everyday risk assessments.

As a trade association, CTPA has been crucial in implement-

ing scientific advancements in practice; facilitating open dialogue between key partners and delivering education to increase confidence in NAMs; and encouraging companies to share their expertise and learnings within industry and with regulators. "Be brave" is the overarching message from CTPA – making NAMs a priority topic in conversations between the scientific community and regulators, to inspire a change in mindset towards their use for chemicals safety assessment by these key stakeholders.

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Microfluidic bone marrow chips as a potential tool for developmental immunotoxicity testing

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Diseases of the immune system have shown increasing prevalence in the last decades among children in developed countries. The developing immune system is more susceptible to toxicants than the adult immune system, raising the question what role potential immunomodulatory compounds play in that development.

The human bone marrow is one of the primary lymphoid organs, where leukocyte development and maturation takes place. Already today, hematologic toxicities can be predicted *in vitro* in a human bone marrow microphysiological system. Human CD34+ hematopoietic stem cells and mesenchymal stromal cells are combined in a scaffold-based microfluidic culture to investigate effects on the innate immune system by tracing differentiation into monocytes, granulocytes and NK-cells by flow cytometry. Also, the combination of the bone marrow model with allogeneic T-cells to incorporate the adaptive immune system into the model is possible with on-chip T cell activation and killing of target cells.

Another potential approach to model the adaptive immune system is a previously developed *in vitro* lymph node model based on monocyte-derived dendritic cells that are embedded together with an autologous lymphocyte population in a dextran hydrogel.

A current limitation of these systems to model the immune-modulatory potential of compounds is that they are built from adult cells and are therefore not exactly mimicking the developing immune system. A promising path forward are iPSC-derived hematopoietic stem cells and their progeny. With these cells, the ultimate goal of building an autologous patient-specific model of the developing hematopoietic system could be achieved.

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Drug metabolism of CYP P450 substrates in dogs and humans: Genetic perspective

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Dogs are frequently used as test animals in both pharmacological and toxicological assessments of new drug compounds. This study is focused on the comparison of metabolism between humans and beagle dogs for a number of selected model drugs, including the prediction of drug elimination through the liver clearance (CLH). Specifically, we focus on determining the intrinsic *in vitro* clearance ($CL_{int,in\ vitro}$) in the metabolic model, here liver microsomes, and revealing the capacity of drug elimination in the selected model drug compounds in the two species.

The preselected drugs chosen to be metabolized by a range of membrane-bound cytochrome P450 (CYP) enzymes in microsome substrates of humans and beagles are caffeine (CYP1A2), mephenytoin (CYP2C19), bupropion (CYP2B6), dextromethorphan (CYP2D6), midazolam (CYP3A4) and repaglinide (CYP2C8). The assay used for estimating the $CL_{int,in\ vitro}$ used was the *in vitro* t½ method, in which $CL_{int,in\ vitro}$ is derived from the mono-exponential slope of a single depletion curve. The extrapolation of $CL_{int,in\ vivo}$ is performed by using physiologically valid scaling factors (SF) and the liver's metabolic capacity.

The obtained results show a high degree of variability in drug elimination metabolism between humans and beagles and depend on the genotype. Human CYP2B6 and CYP2D6 variant carriers are even slower metabolizers. Additionally, there are large differences between beagles and humans (3-4-fold) which can be further amplified by a skewed genetic profile of a highly inbred population of "lab" dogs. The effect of genotypes certainly impacts metabolic capacity making extrapolations from a dog model to humans less reliable.

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Non-invasive methods for the assessment of animal welfare while testing an immunomodulatory drug

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Introduction: Stress assessments in animal studies not only promote ethical responsibility and animal welfare but can also be used to evaluate the side effects of drugs. The aim of this study was to investigate whether the RORγt inverse agonist GSK805 affects the immune system of cholestatic mice without reducing their well-being.

Methods: Bile duct-ligated BALB/c mice were treated with DMSO as a vehicle or GSK805 (30 mg/kg, p.o.) for 14 days. The efficacy of GSK805 was assessed by quantitative PCR. To determine animal welfare by non-invasive methods, body weight, perianal temperature, a distress score, and burrowing as well as nestling activity were determined.

Results: GSK805 significantly (*$p < 0.0001$) reduced the expression of IL-23R in bile duct-ligated mice. Compared to treatment with the vehicle control, GSK805 had no significant effects on mortality, body weight, perianal temperature, distress score, and burrowing as well as nesting behavior of the animals. However, cholestasis induced by bile duct ligation caused a progressive reduction in animal welfare.

Conclusion: According to the study, GSK805 has immunomodulatory properties without affecting animal welfare, suggesting that this drug could be used for the treatment of various diseases without causing major adverse side effects.

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Establishing scientific confidence and application of NAMs for plant protection product development and regulatory registration

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Historically, hazard and risk assessment for plant protection products have relied on implementation of animal-intensive studies. Recent progress in the development of fit-for-purpose and biologically relevant NAMs enable adoption of alternative methods for new molecule development, regulatory registration purposes. NAMs that have been validated and have OECD test guidelines are currently being applied to replace/supplement traditional *in vivo* testing for global registration of new actives and formulations. For many complex toxicological endpoints, a battery of promising NAMs, although do not yet have OECD TG, could be applied for understanding mode of action, assessing key mechanistic events in the development of toxicity, biological relevance, and for better interpretation of data via weight of evidence assessment, while establishing protective and robust point of departure for human health risk assessment. In this presentation, direct and indirect mechanisms of thyroid toxicity are used as proof-of principle, to demonstrate the applicability of various promising alternative methods, including *in silico*, *in vitro*, zebra fish models and omics approaches for toxicity profiling of plant protection products at various stages of molecule development, for stage-gate advancement decisions and for global registration and re-registration purposes. In addition, the significance of compiling well-characterized positive and negative reference chemicals and experiences and key-learnings on implementing multi-organ microfluidic organ systems for thyroid models will be discussed.

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Integration of technological interference into curated HTS data

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Thousands of chemicals have been screened using *in vitro* high-throughput screening (HTS) assays in the Tox21and ToxCast programs, generating millions of data points that can be difficult to interpret and review in detail. Many Tox21/ToxCast assays use luminescence and fluorescence technologies to generate readouts and determine chemical activity; however, test substances can contain substructures that interfere with these technologies to generate false-positive outcomes. To identify these potential false positives, Tox21 includes a series of assays to test for interference between its chemical library and red, blue, and green fluorescence or luciferase luminescence detection technologies. For chemicals that have not been tested in Tox21, InterPred (https://sandbox.ntp.niehs.nih.gov/interferences/) provides QSAR predictions for chemical interference from a model trained on the Tox21 10k library. To increase confidence in HTS data in the Integrated Chemical Environment (ICE; https://ice.ntp.niehs.nih.gov/), we have integrated Tox21- and InterPred-derived alerts for potential autofluorescence interference into the ICE curated HTS (cHTS) pipeline. We first searched the assay technology annotation inventory in the EPA's InvitroDB v3.5 database and identified over 300 assays that use luminescence or fluorescence. We then cross-referenced the chemicals tested in those assays with interferent chemicals identified in Tox21 and InterPred. Warning flags were assigned to any potentially impacted assay endpoint-chemical pairs, and these flags were added to the cHTS workflow. The interference flags in the ICE cHTS pipeline help to further refine the accuracy and context provided for users to interpret cHTS bioactivity calls.

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OphtalMimic: An innovative *in vitro* test for ophthalmic drug products screening

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The necessity of animal-free performance tests for novel ophthalmic formulation screening is challenging for pharmaceutical sciences. In this way, we developed an innovative device that simulates the dynamics and physicochemical barriers of the eye to evaluate ophthalmic drug performance *in vitro*, the OphtalMimic. This novel device has an exposed area with a synthesized structured three-layered hydrogel-based cornea accommodated in a support base, on top of which resides a simulated eyelid, a simulated culde-sac area, and an inlet and outlet for pumping simulated lacrimal flow. The support base is assembled into an engine that constantly moves from 0° to 50°, which also moves the eyelid. The OphtalMimic was challenged by two Fluconazole formulations, a Poloxamer 16% (PLX16) and a Poloxamer 16% + Chitosan 1,0% (PLX16C10), a formulation with higher viscosity and mucoadhesive properties. Each formulation was tested with a 0.5 mL.min-1 simulated lacrimal flow and a 10-minute test time. All the drained liquid was collected, diluted, and analyzed by LC-UV. The OphtalMimic was able to differentiate both formulations draining 72% ±4 and 65% ±3 of the drug in the PLX16 and the PLX16C10, respectively. Also, the relative standard deviation was below 5%, demonstrating the reproducibility of this innovative ophthalmic product performance testing device. The OphtalMimic is useful for comparing formulations, allowing high-throughput screening during the development, research, and quality phases. Our future perspective is to accommodate a reconstructed human cornea in the support base and provide dynamic results regarding drug permeability together with the dynamic performance of tested formulations.

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Development of *in vitro* phototoxicity test method using reconstructed human epidermis (KeraSkin™)

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We are currently conducting a study for the development a "me-too" *in vitro* RhE phototoxicity test method. The method can be used to identify phototoxicity potential of test chemicals following topical exposure of RhE model (KeraSkin™) to the chemicals in the absence versus present of light. The skin irritation test method using KeraSkin™ is already adopted in the *in vitro* Skin Irritation: Reconstructed Human Epidermis test methods (OECD TG 439) and the model's similarity with the Validated Reference Method (VRM) of the TG 498 (EpiDerm™) has been demonstrated. Two lead labs (Ministry of Food and Drug Safety (MFDS) and Korea Testing and Research Institute (KTR)) and three participating labs (Biosolution, Chemon, and KTR) will carry out this study. We have optimized the Standard Operating Procedure for the method and worked on evaluation of transferability and proficiency.

This research was supported by a grant (23214MFDS262) from Ministry of Food and Drug Safety in 2023

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In vitro stinging test using 3D skin model, hiPSC-derived sensory neurons, and microelectrode arrays (MEA)

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Chemical nociception is a type of pain caused by exposure to certain chemicals. In the context of cosmetics, evaluating chemical nociception is important to forestall adverse sensation during product use. Although conventional methods such as the stinging test are commonly used for this purpose, they are not objective assessments; they rely on subjective evaluations by human volunteers, and thus frequently result in inconsistent data.

In this study, we aimed to design an alternative *in vitro* method for the conventional stinging test using human-induced pluripotent stem cell (hiPSC)-derived sensory neurons. We recorded neural activities of these cells upon exposure to chemicals with microelectrode arrays (MEA). In order to better simulate the human skin environment, we also considered a method in which 3D skin model was installed on top of the cultured sensory neurons.

We first verified our method with three known chemical agents: TRPV1 agonist capsaicin, TRPA1 agonist allyl isothiocyanate (AITC), and TRPM8 agonist menthol. Changes in neural responses were detected when hiPSC-derived sensory neurons were exposed to these chemicals.

We next tested our *in vitro* method with fragrance ingredients to explore its application in cosmetics. p-Anisaldehyde, cinnamyl alcohol, and gamma-nonalactone were selected for their ability to cause stinging sensations in human volunteers. These samples also induced changes in neural activities when tested with our *in vitro* assay.

The *in vitro* assay in our study is an ethical alternative to the conventional stinging test and shows promising results for its application in screening cosmetic ingredients.

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Sex matters: Insights from a syngeneic orthotopic model of metastasized pancreatic cancer

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Purpose: The influence of sex as a biological variable on drug efficacy studies in preclinical cancer research is often neglected. This study investigated differences between male and female mice in a syngeneic, orthotopic model of metastasized pancreatic ductal adenocarcinoma (PDAC).

Methods: After orthotopic implantation and intravenous injection of 6606PDA cells into male and female C57BL/6 mice, cohorts were treated either with a combination of trametinib, BKM120 and BI3406 or vehicle control. Wellbeing of the mice was evaluated by analyzing burrowing behavior, body weight, nesting activity and a distress score. In addition, the tumor weight and rate of metastasis were assessed.

Results: Compared to female mice, male mice showed higher rate of metastasis, lower survival and higher tumor weight irrespective of therapeutic intervention. While therapeutic intervention prolonged survival and reduced tumor weight in males, response in females was less apparent. Burrowing behavior, nesting activity and body weight in males were reduced compared to females, while the distress score tended to be higher.

Conclusion: Male and female C57BL/6 showed differences in therapy response, survival and distress in a syngeneic, orthotopic model of metastasized PDAC.

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Phtalox hand sanitizer cream antiviral action against SARS-CoV-2

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Phtalox is a phthalocyanine used to reduce COVID-19 symptoms in mouthwash. We evaluated antiviral capacity of hand sanitizer cream containing Phtalox and permanence time using Reconstructed Human Epidermal model (SkinEthic™ RHE), CCL-81 VERO cells and SARS.CoV2/human/Bra/SP02cc/2020 virus (Genebank MT350282). Experiments were conducted in NB-3 Laboratory following WHO Biosafety regulations in compliance with Good Laboratory Practices. Test product was applied on RHE and viral solution was tested for cytotoxicity. After 30 minutes, supernatant was inoculated into different wells containing Vero cells. Viral solution was stored to quantity SARS-COV-2 by RT-PCR after 72 h incubation in DMEM. Cell culture was fixed and stained with Naphthol Blue Black (Sigma-Aldrich). No cytotoxicity was observed in VERO cells. RT-PCR results showed that both hand sanitizers were able to reduce the viral load by 100% within 5 minutes and 1 hour. After 2 h of application the 0.05% cream showed a reduction of 89%; during 3 and 4 hours a 100% reduction. At 0.25% the reduction was of 91%, 98% and 74% in viral load, at 2, 3 and 4 hours, respectively. The hand sanitizers with Phtalox were not cytotoxic to Vero cells and presented a viral reduction capacity up to 4 hours after application.

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Advances in the use and implementation of 3D human skin-model based genotoxicity assays

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In vitro genotoxicity assays are being accepted as hazard identification tools already for decades in regulatory settings. Positive results from these assays were typically de-risked by follow up testing in animals. This standard *in vitro* genotoxicity testing battery consisting of tests evaluating mutagenicity and clastogenicity/aneugenicity potential has been shown to overpredict hazards in animals and humans. Limitations of these assays thought to contribute to overprediction include the lack of "*in vivo*-like" behavior for key cellular functions like proliferation, morphology, metabolism, gene and protein expression, and function. These features are better reflected in human tissue or organ models which have started revolutionizing toxicological research and have demonstrated potential to serve as direct replacement of *in vivo* tests. Two such higher complexity models we have developed, optimized, and validated two assays for assessing potential genotoxicity of substances with dermal routes of exposure are the reconstructed skin (RS) Comet assay and the RS micronucleus (RSMN) test. Combining these assays enables detection of DNA damage leading to all three types of genotoxic damage (gene mutation, clastogenicity and aneugenicity) and results in excellent predictivity of *in vivo* outcomes. These assays have been accepted into the OECD guideline development program and are currently undergoing formal validation peer-review. Methodological advances of these methods we are currently working on are expected to further increase their utility and support implementation into regulatory schemes. These include automated evaluation via imaging flow cytometry, ability to incorporate liver metabolism, and an adaptation of the RSMN assay that enables the detection of photo-genotoxins.

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A bioethical engagement with the CCAC revised ethics document

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The Canadian Council on Animal Care (CCAC) is revising their basic ethics document and this presentation analyzes the proposed revisions from a bioethics perspective. To accomplish some of this, the draft document will be briefly compared with the CCAC's current ethics statement as well as some favoured ethics principles in human research ethics. Then I will devote most of my time to discussing how the revised document promises to advance discussions on some pressing ethics issues in the use of animals in science. A significant advance over the CCAC's current ethics statement is the explicit embrace of bona fide core ethics principles (at the time of writing, Non-maleficence, Sufficient Benefit, and Respect). There are also principles of application that serve to illustrate how the core principles can be applied in practice. Taken together, they can be reasonably seen to reflect changes already afoot in animal research ethics, particularly the ethical importance of rehoming or retiring "surplus" animals. Moreover, they also effectively support re-seeing killing as a harm – which remains more controversial in the relevant science communities than it should be. As this is a bioethical engagement with the draft document, I will also speak to what is yet underdeveloped in the revised document and how future revisions can further advance animal research ethics in Canada. A serious but common ethics lacuna in scientific animal use is adequately dealing with non-anthropocentric justice issues. I will argue that several issues surrounding rehoming or retiring animals shows why justice must enjoy greater prominence.

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Towards a proximal tubule microphysiological system for antisense oligonucleotide safety testing

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Antisense oligonucleotides (ASOs) enable the modification of the expression of clinically relevant target mRNAs. The PCSK9-targeting ASO SPC5001 was terminated in a clinical phase 1 study due to dose-related tubulotoxicity, which the preceding preclinical toxicity studies performed in mice and non-human primates failed to predict. In the current study, we evaluated whether ASO-related target gene knockdown and tubulotoxicity could be observed using the HUMIMIC Chip4, a microphysiological system (MPS) that enables the long-term co-cultivation of a proximal tubule model with additional organ models of interest. This constitutes a highly physiologically relevant model that can be used to assess organ-specific toxicity, targeting and exposure routes in future studies.

Here, we seeded primary hRPTECs into the HUMIMIC Chip4 and cultivated them using a defined fluid flow for 28 days. Following a 7-day equilibration phase, cells were exposed to the nephrotoxic ASO SPC5001 or a control ASO on days 7, 14 and 21, mimicking a weekly clinical dosing regimen. SPC5001 treatment resulted in an efficient target gene knockdown with a 10-fold lower PCSK9 expression on day 28 compared to the controls, as well as increased lactate secretion and an increased number of dead cells.

In conclusion, this proximal tubule MPS presents a promising tool for future ASO toxicity studies with the possibility of extending the cultivation and exposure period, incorporating additional organ models or utilizing PBPK modelling and *in vitro-in vivo* extrapolation to enable clinically relevant predictions of ASO toxicity.

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Obstacles, solutions & recommendations: A knowledge agenda for the transition towards animal-free science

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Many new therapies, despite being effective in laboratory animal models, work less well or even not at all in humans. Therefore, there is a growing need for the development and application of animal-free methods, which effectively translate to human (patho) physiology. The transition from an animal-focused to human-centred approach is a long-term process, as it runs counter to past habits and existing interests, and requires the trust in animal-free methods to grow. To identify the challenges that impede this transition, we consulted a large number of academic and commercial researchers, regulators, students and lecturers from various Dutch universities and institutes who have extensive experience with laboratory animals and/or animal-free innovations. This resulted in a prominent knowledge agenda, in which we identified various obstacles and solutions at a scientific and technical level, but also at an educational, political, societal and cultural level. Moreover, we formulated many specific recommendations addressed to a large variety of stakeholders, enabling a broad approach to boost the transition.

By its design, this knowledge agenda is both widely supported and far reaching. Although we focus on developments and opportunities in the Netherlands, our approach as well as the ensuing results may also be helpful for other countries, as the challenges and solutions are obviously influenced by international developments. We therefore hope that the entire community working on animal-free innovations will relate to this knowledge agenda. With the right initiatives, we believe we can inspire many stakeholders to stimulate this transition towards more human-oriented, animal-free science.

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How to train the trainer: Influence of education on positive reinforcement training

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Experimental procedures with farm animals are often associated with restraining techniques. Both may cause stress in farm animals, influence results and bring about a risk for occupational accidents. Using positive reinforcement training in this context can reduce stress in animals and therefore implement refinement according to the 3R Concept. Trainer skills may influence the success of positive reinforcement training. To be successful and effective in respect to invested time and resources, animal trainers must be consistent, precise, and structured in their training technique. However, the potential influence as well as the required education of animal trainers are rarely described in literature, but necessary for implementation of positive reinforcement training as refinement measure.

To investigate the effect of two educational programs on animal trainers, we compared the training success of two groups of participants in training goats within two weeks for a behavior allowing simulated venipuncture. One group was educated in a two-day workshop while the other was provided with specific literature for self-instructed learning. Training success was greater in the workshop group ($p = 0.004$). In addition, 73% of the participants in the workshop group stated that they could completely implement the content of the workshop in training their goat, in contrast to 13% in the self-instructed group. Our results indicate that more intensively educated trainers can train animals more successfully. In conclusion, if animal training is implemented as refinement, potential animal trainers such as animal caretakers or technicians should receive instructions for the respective training.

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Overcoming the industrialization challenges for *in vitro* absence of toxin testing in tetanus vaccines

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When using animals, GSK follows scientific principles to prevent or minimize pain and distress before, during, and after experimental procedures to develop innovative products and perform the mandatory release of vaccines.

The majority of animal testing in GSK is linked to quality control of vaccines. By developing and applying non-animal methods, GSK has reduced the utilization of animals by 60% in the last seven years and we aim for 75% by 2025.

Recently, the European Pharmacopoeia adopted 16 revised monographs on tetanus vaccines, including the suppression of three animal tests. Despite this progress, the *in-vivo* Test for absence of toxin remains in place. Replacement of this test by an *in vitro* method is desirable from animal welfare and innovation perspectives. Although expected to be eventually replaced, it can take years and even decades to realize. Therefore, GSK´s 3R program initiative is to proactively replace it with the BINACLE (binding and cleavage) ELISA, developed by the Paul-Ehrlich-Institut.

The BINACLE has demonstrated its suitability in an international collaborative study (BSP136) as a more sensitive *in vitro* alternative to the Test for absence of toxin for tetanus neurotoxins (TeNT). This method relies on non-commercially reagents as the polyclonal antibody specific for cleaved synaptobrevin. Further not all reagents comply with EMA guidelines and GMP standards, which is a critical requirement for the assay´s industrialization as release test. To overcome this challenge, GSK has developed and evaluated the suitability of anti-Syb2 monoclonal antibodies for their use on the BINACLE assay under the required quality standards.

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Assessment of animal-free methods to substitute *in vivo* potency assays for DTaP vaccines

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When using animals, we follow scientific principles to ensure that we prevent or minimize pain and distress before, during, and after experimental procedures to develop innovative products and perform the mandatory release of vaccines. Historically the majority of animal testing in GSK is linked to quality control of vaccines making it a key element to reduce animal use by developing and applying non-animal methods (NAM). GSK has reduced the utilization of animals by 60% in the last seven years and we aim for 75% by 2025 in Vaccines Quality. The last major animal tests in quality control are the potency assays for Diphtheria, Tetanus and acellular Pertussis (DTaP) vaccines. Different DTaP potency animal-free analytical technologies have been developed by public partners in the European IMI2 Vac2Vac project, finalized in 2022 and in which GSK participated. The most promising methods assessing antigen content and antigenicity were ELISA, Luminex, MSD and LCMS. GSK has further evaluated the feasibility of these methods on seven GSK products in terms of technical, GMP and regulatory requirements. In this talk, we will present our results on (i) immunoassays comparison (ELISA, Luminex and MSD), (ii) standard definition with DTaP lyophilized materials, (iii) antigen age impact on the assay performance and (iv) preliminary *in vitro* and *in vivo* comparison generated to demonstrate the suitability of animal-free *in vitro* methods to substitute the current *in vivo* potency assays.

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Refinement: The impact of peri-operative analgesia on neuropharmacological outcomes in rodent models of chronic pain

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Chronic pain affects approximately 20% of the population, and since currently prescribed analgesics are ineffective in three quarters of these individuals, identifying novel pharmacotherapeutic targets is imperative. Multiple mechanisms contribute to pain and animal research is vital in order that we may expand our knowledge regarding the underlying causes. This involves the use of surgical procedures that ethically require analgesia. However, since basic pain researchers wish to measure pain-related behaviours, and on the assumption that peri-operative analgesia would impact neuropharmacological outcomes, animals are often denied peri-operative analgesia. This means that animal welfare in the acute post-surgical period is not optimally considered. Thus, the aim of this project is to refine the use of peri-operative analgesic administration for rodents undergoing surgeries by investigating its impact on electrophysiological and behavioural outcomes. Pregabalin (3 mg/kg) will be administered to rodents undergoing spinal nerve ligation surgery preceding a battery of behavioural tests and electrophysiological analysis. My pilot data shows that in spinal nerve ligated rats, a single dose of pregabalin administered 15 minutes prior to surgery does not significantly change baseline on spinal neuronal responses 14-16 days post-surgery, Behavioural data show that animals who did not receive any peri-operative analgesia manifest mechanical hypersensitivity on day 1-2 post-surgery compared to those who received a single dose of pregabalin. If post-operative pain behaviours can be reduced in the acute period, while neurological activity is unchanged in the chronic period, researchers should be willing to re-address the importance of perioperative analgesia in rodent models of chronic pain.

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Bringing quality control and batch release testing of biologicals into the 21st century

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The 3Rs (Replacement, Reduction and Refinement of animals in research and testing) have gained significant traction globally as a framework to support the best, most innovative science that reduces our reliance on animals and improves their welfare where they are still required. This is especially true right now in the quality control and batch release testing of biological products where large numbers of animals are used globally each year. The assays used in these tests have changed little since they were first introduced, but there now exists a swell of support for change and to explore how recent advances in science and technology can be applied to this field to bring it into the 21st century.

Over the last decade significant strides have been made to apply non-animal approaches in the quality control and batch release testing of biological products and to remove obsolete tests such as the general safety test. However substantial challenges remain

to the global adoption of these by some regulatory authorities and manufacturers, which continues to drive the use of animals despite the availability of alternative approaches. This session will explore currently available non-animal methods, the challenges to their use and how by working as a community may begin to address these to support the wider adoption of the most scientifically relevant testing approaches available.

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A spoon full of sugar? Refining mouse housing and experimental procedures with the help of rewarding treats

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Millions of research mice are used annually, often to model aversive conditions (e.g., cancer, chronic pain, anxiety). Yet outside of unavoidable distress to meet research objectives, conventional housing, handling, and experimental procedures further compromise mouse wellbeing. Numerous evidence-based refinements can address these issues (e.g., providing well-resourced, "enriched" environments instead of barren cages; non-aversive cup/tunnel handling instead of tail handling), but constraints on time and resources often present barriers to implementation. Here, we show three ways in which rewarding treats can be used to improve mouse well-being through refinements and assist researchers. The first addresses challenges faced by researchers aiming to house mice in large, well-resourced cages in which catching animals can be hard: presenting a simple protocol in which mice are readily trained to enter a designated handling area of the home cage. This facilitates catching and handling mice in well-resourced environments and enhances abilities to detect problems during daily health checks. Next, we demonstrate the value of high value food rewards (paired with non-aversive handling and ethological designs) for cognitive testing. This approach allowed mice to rapidly meet learning criteria in a complex cognitive task (judgement bias), without punishment or aversive stimuli. Finally, we present a refined mouse anesthesia protocol, reducing pain and restraint stress through administering a voluntary oral analgesic pre-medication in condensed milk (followed by a subcutaneous injection to induce anesthesia, as opposed to intraperitoneal). These simple protocols can be readily implemented across diverse research areas, improving animal welfare while meeting the needs of researchers and technicians.

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Establishment of a human-based *in vitro* model for synaptic plasticity

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Synaptic plasticity is characterized by the ability of neuronal networks to modify their synaptic connection strength and is considered an important feature for learning and memory. Long-term potentiation (LTP) serves as a model for synaptic plasticity and is represented by the persistent strengthening of neuronal synapses in response to an electrical stimulus. To date, LTP has been evaluated using *in vivo* mouse models, brain slices, or non-organized neuronal cultures. As an alternative to *in vivo* animal models, this study sought to develop a serum free *in vitro* model to assess synaptic connectivity and neural network integrity associated with cognitive function using human induced pluripotent stem cell (hiPSC) cortical neurons and human-on-a-chip (HoaC) technology. Human iPSC-cortical neurons were cultured on microelectrode arrays (MEAs) with photolithographic patterned surfaces to encourage paired neuronal network formation. To evaluate LTP, an electrical stimulus was performed on only one neuronal population within each paired network and enhanced activity was assessed in the connected population. The enhancement in neuronal activity was hypothesized to be due solely through synapse connectivity, which was confirmed by the inability to induce and maintain LTP activity after blocking AMPA and NMDA receptors. This study validated a hiPSC-based, serum-free HoaC system capable of assessing neuronal synapse integrity and evaluating LTP solely through synaptic connectivity. The establishment of this system enables further use for the investigation of neurodegenerative diseases for preclinical drug assessment.

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Current state policy of animal testing in cosmetics in Latin America

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Latin America lags behind the EU by ten years of legislation regarding animal testing for cosmetics. Awareness over this issue has raised over the past six years, yet there is currently no official information on the number of animals used for cosmetic testing and the development of alternative methods laboratories is incipient and lacks state support.

There are 33 countries in Latin America, with a market size estimated at 51 billion dollars in 2020, in which Brazil, México and Chile represent 74% of this market. An increase of cruelty-free brands has been observed in the last decade. In Chile, México and Brazil for instance, between 2019-2023 there was a 53% growth in cruelty free brands available in each country. Furthermore, campaigns like #SaveRalph had a significant impact in Latin America and support in countries like Brazil and México went viral with 3 million signatures, creating a social phenomenon.

So far four countries have legislated to ban animal testing for cosmetics and several bills have been introduced. However, current legislations are not aligned within the international industry language and have legal gaps that could allow bypassing.

There is an opportunity to develop a roadmap to promote an alignment between industries, government entities, and civil associations within the region, to work on a common legislative language and implementation path that allows to advance to an effective animal-free policy for cosmetic testing. There is also a gap for incentives on NAMs research, which such a roadmap would help to close.

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Developing a physiologically relevant small intestinal model for drug assessment

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In-vitro small intestinal modeling has proven to be a valuable tool in studying how pharmaceuticals and other chemicals affect *in-vivo*-like human gastrointestinal (GI) tract physiology. Animals tend to be the model of choice as *in vitro* models lack many basic properties of human GI tract function. Many current *in vitro* models employ immortalized cell lines consisting of Caco-2 enterocyte-like cells and mucus secreting HT-29 cells, but these cell lines offer limited metabolic activity, which is an important factor for evaluating effectiveness of oral dosing. To address this issue, we compared different iPSC-derived GI tract models consisting of organoids and monolayer enterocytes with the conventional Caco-2/HT-29 model. Our results suggest that of the different models tested, the iPSC-differentiated enterocyte monolayer model is the most promising approach. Immunocytochemistry and flow cytometry results confirmed the expression of villin and mucin-2, which is specific to enterocytes and goblet cells, respectively, and makes up a majority of the small intestinal epithelia. To confirm the presence of the CYP3A4 metabolic enzyme activity we performed commercial and in-house probe-based quantification assays, which showed levels of enzymatic activity up to 3.2% metabolite to parent conversion. Viability assays showed that cell viability increases by 10% 7 days post-enterocyte maturation. With its promising *in-vivo*-like properties, this iPSC-derived model can be used as a first pass metabolism model for drug screening and testing. In combination with other organ modules on-a-chip, it can help model oral drug delivery profiles and pave the way for patient specific testing and reduce animal utilization.

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The potential of multi-organ microphysiological systems in drug development

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The advancement of microphysiological systems (MPS) has allowed for its augmented use in drug development. However, additional work is needed to make these models an effective alternative to animal testing. We have constructed a gastrointestinal (GI)-liver-blood brain barrier (BBB)-central nervous system (CNS) MPS model to test drugs targeting neurodegenerative disease (NDD). To validate our model for drug efficacy and transport studies, we used a cortisol-induced stress model of the CNS based on long-term potentiation (LTP) to test the effects of a vehicle control and cannabidiol (CBD) dose administered "orally" (within the GI) into the system. CBD is an established stress-reducing chemical known to affect endocannabinoid receptors. Real-time TEER measurements of the GI and BBB showed barrier integrity maintenance throughout the 48-hour experiment. Albumin production (~3 µg/mL) and CYP3A4 enzyme activity (~22 pmol D-Luc/h/10^6 cells) by the liver and viability of all organ constructs was confirmed at 48 hours, intimating proper organ health and function post-dose. To confirm CBD's beneficial effect on CNS stress reduction, the presence of free anandamide (AEA) in the system and LTP maintenance in the CNS was examined. Compared to undosed controls, systems dosed with CBD indicated higher levels of free AEA in the CNS at 24 and 48 hours. Additionally, there was a significant rescue of LTP maintenance at every time point, indicating reduced stress levels in the CNS. These findings demonstrate the capabilities of our system to be used as a drug-testing platform, especially for NDD.

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Development of a 3D human chronic wound model using animal-free products

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Chronic wounds represent scenarios where the acute wound healing response is impaired, burdening both patients and healthcare systems. Rodent models are often used to assess novel wound healing therapeutics; however, these models have limitations in their translation to human wound healing scenarios due to vital differences in skin architecture and wound healing cascade. We have developed a 3D organotypic chronic wound model as an alternative to animal models, to represent more closely the complex human *in vivo* scenario.

This novel 3D organotypic model comprises human chronic wound derived fibroblasts and epidermal keratinocytes, cultured over 14 days at the air-liquid interface, resulting in epidermal differentiation. This 3D organotypic model underwent H&E staining, along with immunostaining for key components of the epidermal and dermal layers, including keratin 10, keratin 14, involucrin and fibronectin. Use of chronic wound derived cells is key for accurately representing the impaired wound healing environment, due to phenotypic differences in cell function, compared to healthy dermal cells.

Most cell-based research utilises animal-derived products, whereas this model is cultured using entirely synthetic, animal-free products, resulting in better repeatability in the studies, due to the batch variability associated with animal product use. Many industries and funders are already expressing an interest in replacing animal use in research, but a viable option is required to sufficiently replace currently used models. This novel 3D chronic wound model will more accurately represent the human wound healing scenario and without the ethical considerations associated with rodent models and animal-product use.

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The assessment of phototoxicity using the reconstructed human epidermis, KeraSkin™: An *in vitro* evaluation of the phototoxic possibility of chemicals

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The increasing demand for tighter regulation of animal experiments has prompted the need for the development of alternative *in vitro* models to evaluate the phototoxicity of cosmetics and new drugs. This study aims to investigate the ability of the reconstructed human epidermis (RhE), KeraSkin™, to evaluate the phototoxic possibility of chemicals after topical treatment. Six proficiency chemicals were selected from the OECD test guidelines (OECD TG 498) to assess the phototoxicity using KeraSkin™. The UVA sensitivity of KeraSkin™ was determined, and 6 J/cm^2 was selected as the UVA irradiation dose according to OECD TG 498. The phototoxicity prediction was assessed by comparing the viability of UVA-irradiated and non-irradiated KeraSkin™ after the application of 3 phototoxic and 3 non-phototoxic chemicals using the MTT assay. As a result, chlorpromazine, anthracene, and bergamot oil were evaluated as phototoxicity and sodium dodecyl sulphate (SDS), octyl salicylate, and 4-aninobenzoic acid (PABA) were determined as non-phototoxicity. The results of this study indicated that KeraSkin™ is a suitable model for evaluating the phototoxicity of cosmetics and drugs, and a useful tool for establishing *in vitro* phototoxicity tests using domestic skin models.

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Developmental toxicity test by long-term signal disruption observation using human iPS cells

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Developmental toxicity of chemicals has been assessed with laboratory animals. However, this approach involves several challenges, including species differences, the use of large number of animals, and costs. To satisfy the 3Rs principle, there is a need for the development of alternative *in vitro* approaches that can accurately evaluate the human teratogenicity in a high-throughput manner. We have previously developed a human iPS cell-based assay for predicting the developmental toxicity of chemicals using the FGF signal disruption [1]. This assay successfully detected chemicals listed in the European Centre for the Validation of Alternative Methods (ECVAM) *in vitro* developmental toxicity study. A notable feature of this assay is that disruptions occurring at various time points can be detected by integrating signal disruptions over time [2]. However, because the measurements were conducted by manual handling, the number of time points were limited. In this study, we examined whether the outcomes were further improved by continuous monitoring of signal disruption using an automated luminescence equipment. This approach could be a promising tool for the initial screening of developmental toxicants.

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HUMIMIC-InHALES: A human-relevant aerosol test platform for systemic exposure studies

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Current aerosol exposure systems suffer from the limitation that they expose only discrete parts of the human respiratory tract in static *in vitro* cultures to a fraction of a complex aerosol. This limits the predictive power of the generated data for respiratory and systemic human effects caused by such complex aerosols.

Philip Morris International (PMI) developed the Independent Holistic Air-Liquid interface Exposure System (InHALES) as a mechanical replica of the whole human respiratory tract. It perfectly matches the architecture and respiratory characteristics of the human respiratory tract, including three relevant respiratory tract compartments [1]. PMI engineered InHALES to implement TissUse's proprietary microphysiological HUMIMIC multi-organ-on-a-chip platform. A novel HUMIMIC chip for plug-and-play insertion into InHALES was developed to maintain and culture a human cell culture insert-based lung model with other organ equivalents (e.g., the liver). The use of new materials significantly reduced the absorption within the chip and allowed for testing of hydrophobic compounds. We have demonstrated the lung model's integrity and viability using CellTrace™ Calcein Red-Orange AM and CellTox™ Green staining. The airway cultures in the HUMIMIC chip are subsequently exposed to physiological aerosols generated by the InHALES.

With its combination of aerosol test system and cutting-edge microfluidics, HUMIMIC-InHALES supports the development of any systemic tests for aerosol exposure, including acute and chronic toxicity and long-term treatment efficacy.

Reference

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An *in vitro* microfluidic model of the human cardiovascular system for use in pharmaceutical screening applications

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Cardiovascular disease is the leading cause of death worldwide; however, no regulatory assays are currently available to predict drug/chemical-linked effects on atherosclerosis (gradual narrowing of the arteries). We use a BioFlux™ microfluidic system to model blood vessels *in vitro*. This platform has potential for scalability and sustainability due to utilization of the commercially available BioFlux™ device. Primary human aortic endothelial cells lining microfluidic channels are exposed to shear flow, mimicking blood vessel physiology and blood flow through the vasculature. Using this technology, we have developed physiologically-relevant *in vitro* assays that model atherosclerosis, providing a bridge between *in vitro* and *in vivo* experiments. One such assay evaluates monocyte-to-endothelial cell adhesion and cytokine release in response to test compounds, thus modelling an important early stage of atherosclerosis and predictive of *in vivo* inflammatory outcomes. The assay is relevant to multiple industries (e.g., pharmaceutical, food, chemical) as a safety assessment tool for prediction of atherosclerotic risk.

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ChemMaps.com V2 – Exploring the environmental chemical universe

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Access to computationally based visualization tools to navigate chemical space has become more important due to the increasing size and diversity of publicly accessible databases and associated compendiums of high-throughput screening (HTS) and other descriptor and effects data. Construction of visualization tools relies on complex projection techniques using molecular descriptors. However, application of these techniques requires advanced programming skills that are beyond the capabilities of many stakeholders. Inspired by the popular Google Maps application, we de-

veloped the ChemMaps.com webserver (https://sandbox.ntp.niehs.nih.gov/chemmaps/) to easily navigate chemical space. The first version of ChemMaps.com enabled users to browse and visualize a space of 2,000 FDA-approved drugs and over 6,000 drug candidates from the DrugBank database (https://www.drugbank.ca/). The chemical space of ChemMaps.com V2, released in 2022, includes approximately one million environmental chemicals from the EPA Distributed Structure-Searchable Toxicity (DSSTox) inventory. ChemMaps.com V2 incorporates mapping to HTS assay data from the U.S. federal Tox21 research collaboration program, which includes results from around 2,000 assays tested on up to 10,000 chemicals. ChemMaps.com V2 users can now visualize chemical activity both by assay and target directly on the map and compare chemical spaces occupied by active and inactive chemicals. ChemMaps.com V2 also has new navigation options, including an on-the-fly distance measurement between two chemicals selected on the 3D map, a map screenshot button, and customizable color mapping based on chemical properties.

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Applying *in silico* toxicity models across the Tox21 chemical universe

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In day-to-day life, people are continuously exposed to many chemicals through different exposure routes. Ideally, regulators will leverage all available toxicity information to make regulatory decisions on chemicals that will protect human health. Traditional toxicity testing relies on *in vivo* methods that are time-consuming, resource-intensive, and of questionable relevance to humans. Many available computational models can be applied to predict human toxicity for research and regulatory purposes to reduce time and resource expenditure. The goal of this collaboration was to apply and benchmark such models to the Tox21 chemical set. This set comprises approximately 10,000 chemicals including drugs, consumer products, and pesticides that have been tested in high-throughput screening assays in the U.S. Tox21 program. We applied three models to this chemical set to predict carcinogenicity and drug-induced liver injury. DeepCarc and DeepDILI are deep learning models that integrate five conventional machine learning algorithms into a neural network to generate probabilistic predictions for carcinogenicity and liver injury, respectively. We also applied carcinogenicity and mutagenicity models from the JANUS project (https://www.vegahub.eu/portfolio-item/janus/). We evaluated the confidence of each prediction as well as characterizing each model's applicability domain. Performance of all models was compared and physicochemical and structural properties of predicted active chemicals were defined. Our results suggest that these computational models can be used to rapidly screen large chemical libraries to prioritize potentially hazardous substances for further evaluation.

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Expanding PBPK modeling to predict chemical distribution in brain and adipose tissues

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To facilitate decision making in drug discovery and risk assessment, physiologically based pharmacokinetic (PBPK) modeling approaches are being developed for high-throughput applications. Most existing open-source PBPK models can predict chemical concentrations in major body compartments including blood, liver, kidney, and gut. However, to help assess specific toxicological effects such as neurotoxicity, models need to predict chemical distribution to compartments like the brain that have complex structural features and may be exquisitely sensitive to chemical exposures. In particular, understanding whether a chemical can cross the blood-brain barrier and incorporating that in a PBPK model is important for predicting and accurately assessing its potential neurotoxicity. Additionally, adipose tissue plays a critical role in toxicokinetics by acting as a storage compartment for lipophilic chemicals and a source of continuous internal chronic exposure as the chemical is released. However, this tissue is often not included as a separate compartment in existing PBPK models. Predicting the concentration of chemicals in adipose tissue by leveraging lipophilicity predictions from QSAR models (e.g., OPERA) can provide valuable information on the likelihood of chemical bioaccu-

mulation. This presentation highlights our work to expand existing PBPK models to quantify chemical concentrations in brain and adipose tissues. Case studies will be used to showcase the functionality of these added compartments and explore their potential future implementations in the field of predictive toxicokinetics.

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Tackling post-transplant lymphoproliferative disease: A cutting-edge platform for Epstein-Barr-virus specific T-cell fighters

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Immunosuppressed patients after solid organ transplantation (SOT) are at high risk to develop malignancies. Post-transplant lymphoproliferative disease (PTLD) is one of the most severe complications after SOT, manifesting in the lung in up to 20% of the cases and involving treatment with highly toxic agents.

Adoptive anti-viral T-cell therapy represents a novel, less toxic therapeutical approach, which has shown promising results for PTLD. However, to confirm the safety and efficacy of these EBV-specific T-cell products for clinical translation, we are in urgent need of a suitable human-based test platform overcoming the non-physiological 2D cell culture systems and animal models.

We had the unique chance to set up a human co-culture platform of patient derived 3D lung organoids and patient derived EBV-transformed lymphoblastoid cells (LCLs) mimicking PTLD in the lung, as we have access to primary lung tissue and blood samples from the same patient. In order to generate this platform, we already implemented a co-culture of primary lung organoids and corresponding autologous LCLs. We have found a suitable co-culture medium and investigated LCL interaction and infiltration into the organoid. Patient-specific EBV-reactive T-cell product manufacturing is established in a GMP compatible manner, and we are now integrating the antiviral T-cell products into the platform for testing safety and efficacy. This autologous, human-derived platform has the potential to provide valuable preclinical data on the functionality and safety of EBV-specific T-cell products, may lay the basis for testing other specificities and will pave the way for clinical trials without extensive animal testing.

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Leveraging AOPs to enhance decision making on chemical safety across all stages of the development pipeline

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As we progress in the journey towards assessing chemical hazards without relying on animal testing, integration of data from various new approach methodologies (NAMs) becomes increasingly central to inform decision making on chemical safety. Frameworks built around adverse outcome pathways (AOPs) can facilitate the integration of data from both traditional and emerging methods, allowing for comprehensive assessments which can provide mechanistic insight, identify data gaps, refine testing strategies, and assess confidence in the overall conclusions. To illustrate those benefits for screening and regulatory applications for developmental/reproductive toxicity (DART) and carcinogenicity, respectively, relevant assays and (Q)SARs were annotated to expert-curated AOPs. The resulting network contained 61 AOPs and was associated with information for over 200,000 unique compounds. To evaluate the DART screening tool, 199 compounds from a zebrafish dataset were profiled, where the application of QSAR models and similarity searches in tandem demonstrated increased sensitivity towards predicting toxicities compared to using a single method in isolation. For the carcinogenicity application, a workflow based on the requirements of the ICH S1B addendum was created. Profiling two carcinogenicity datasets demonstrated the workflow provides good sensitivity, identifying 83% on average for known human and animal carcinogens. In both settings, expert review can take advantage of having available evidence grounded alongside mechanistic information. AOPs through the unification of toxicity knowledge and data can support assessors in a range of decision-making settings through continued contextualization and organization of the growing evidence base that is occurring with the emergence of NAMs.

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Dynamic models replace animals to study long-term effect of additives on rumen fermentation

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The traditional continuous culture system (CCS) was designed to evaluate effects of feedstuffs on rumen fermentation. Active ingredients such as yeasts probiotics need a longer adaptation period which *in vitro* tools often lack. The aim of this study was to modify the CCS to mimic efficiently a 17-day fermentation. The system was composed of 24 Duran fermenters fitted with 2 side arms to evacuate digesta and allow sampling for analytics. The cap was equipped with automatic monitoring pH, redox potential (Eh) probes. The trial involved a 7-d stabilization period and a 10-d of supplementation of live yeasts and the fermenters were fed pellets twice a day. After 17-day of fermentation, CCS adapted rumen fluid was used to evaluate fiber degradability of wheat straw in Daisy Incubator II. Data were processed by GLM repeated and univariate model. Results showed that CCS imitated closely *in vivo* rumen pH & Eh kinetics having nadir pH values of 5.8 and 5.5 after first and second meals respectively. Live yeast strengthened the anaerobic character of the milieu by lowering Eh (- 8 mV) when compared to no yeast. Degradability results showed a significant increase in DM (+ 2.9%), OM (+ 3.1%), NDF (+ 5.5%) and ADF (+ 4.8%) degradation in the presence of yeast. CCS proved to simulate rumen fermentation after a 17-day period. Combining these two *in vitro* systems showed valuable results especially when studying probiotics supplemented on a long period.

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Alternative models to better understand ruminant digestive physiology

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Ruminant digestive physiology is still a major concern for animal scientists as these animals that digest fibers to produce animal protein (milk and meat) for human consumption, produce also some undesired and polluting excreta. In this field, models that replace ruminants have been largely used and showed interest in evaluating raw materials for better feed digestibility, screening new products for rumen improvement, health, and environmental issues. At Phileo by Lesaffre, a dedicated team has been pushing boundaries of science to design 3 innovative models that can replace, reduce, and refine animal experimentation. The first model (Model 1) mimics rumen fermentation in bioreactors allowing to better understand ruminal degradability of forages *in vitro*. A dual flow model combined with a Daisy Incubator showed reliable degradability values to estimate milk performance at animal level. The second model (Model 2) seek to underpin immune challenges from a blood sample. Readouts such as ROS (Reactive Oxygen Species) or gene expression after cell restimulation helped in determining pro- and anti-inflammatory responses in *ex-vivo* conditions. The third model (Model 3) aims at detoxifying the rumen from mycotoxins. An experimental design with ANKOM Gas Production technology coupled with fiber degradability allow a better insight on how additives (pro- and post-biotics) strengthen the animal resistance to mycotoxins. As a matter of fact, Models 1 and 3 replace cows usually utilized for rumen explorations and toxicology studies. Model 2 reduces and refines protocols to use less animals with a better care and attention given when sampling.

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Determining regulatory endpoints for an agrochemical without bioassays

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Sufficient data to establish regulatory endpoints for cyclobutrifluram, a new mitochondrial complex II succinate dehydrogenase inhibitor (SDHI), were available from studies up to and including 90-day repeat dose toxicity studies. The chronic/carcinogenicity assays in rats and mice were not needed to address human safety or conduct risk assessments.

Cyclobutrifluram had low toxicity, and was not genotoxic, neurotoxic, immunotoxic, nor a developmental or reproductive toxicant. Toxicity findings were typical of SDHI's: liver weight increases and centrilobular hepatocellular hypertrophy in mice, rats and dogs; thyroid follicular cell hypertrophy in rats; elevations in liver enzyme activity in rats and mice; with no additional or unique findings. The lowest no observed adverse effect level in studies up to 90 days duration was 51 mg/kg/day in rats. In a two-year study in rats at doses up to 23 mg/kg/day, body weight/body weight gain decrement were the only findings. The lowest no effect level in mice after 18 months of dietary exposure was

14 mg/kg/day, due to an equivocal increase in hepatocellular carcinomas, in male mice at 48 mg/kg/day. Since the test item was clearly demonstrated to induce CAR activity and transient cell proliferation, these tumours are not relevant to human risk assessment.

A point of departure for risk assessments of 51 mg/kg, adjusted by 3-fold for duration, provided an equivalent endpoint to the NOEL in the mouse carcinogenicity study. A human health protective risk assessment can be conducted based on studies up to 90 days in duration, supported by evaluation of similar chemicals.

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Development of the type 1 diabetes model islet *in vitro*

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Multicellular spheroids are thought to be useful to recapitulate organ functions *in vitro*. Typical spheroids are made only from cells, however extracellular matrices (ECMs) are also important factors of healthy and diseased organs. For example, hyaluronic acid (HA) is deposited in the pancreatic islets in type 1 diabetes (T1D). Here, we report a method to fill spheroids composed of islet-cells with HA and demonstrate the effect of the pathological ECM on islet functions.

We isolated primary mouse islet cells and suspended in culture medium at 8e6 cells/ml. To fill HA into the spheroids, the cell suspension medium dissolving HA was prepared. We injected 1 µl of the cell suspension with or without HA into the 3% MC medium to aggregate the cells. After 10-30 min, the injected cells (and HA) were concentrated, and stable spheroids were formed after 24 h in the MC medium. The spheroids were isolated from the MC medium and used for subsequent assays.

Compared with normal islets, HA-loaded T1D model islets showed less amount of glucose stimulated insulin secretion (GSIS). In the T1D model islets, ATP synthesis and Ca^{2+} uptake after stimulation with glucose were reduced. The filling of heparan sulfate, an ECM detected in healthy islets, were not inhibited the GSIS. These results indicated a possibility that HA is not only an inflammation mediator in T1D but also a factor of GSIS inhibitor. We believe that this method filling spheroids with ECMs can be applied in various studies.

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Review of complex *in vitro* models representing the pulmonary system

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In the past most *in vitro* models including those representing the pulmonary system were based on a single cell-type and in case for the pulmonary system even cultured under submerged conditions, which is not the physiological situation for lung cells. In recent years complex models using more than one cell type in a 3D orientation were developed and have become widely applied. Models relevant for the lung are commercially available and can represent the whole respiratory system from the nasal cavity to the alveolar barrier. These commercial models can be based on primary cells or cell lines with the inherent advantages and limitations of the two different approaches.

Endpoints studied in the various models range from irritation, inflammation, respiratory sensitization, to carcinogenesis and cells can be incubated in static, pressurized or cyclic systems. Dosimetry is an important factor and may reach its limits in MPS systems.

Overall, models should be as complex as necessary to mimic the physiological responses studied and as simple as possible to reach that goal while allowing dosimetry and measurement of relevant endpoints.

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A systematic review on pollutant induced cardiotoxicity: Providing a toxicological foundation for NAM development and regulatory acceptance

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Cardiovascular disease (CVD) is the leading cause of mortality worldwide, and CVD incidence is expected to rise with the aging population. Lifetime exposure to environmental chemicals

has been linked to the development and severity of CVD. The EU-Horizon 2020 ALTERNATIVE project aims to develop an animal-free test platform for improved regulatory cardiotoxicity assessment of chemicals. Systematic reviews (SRs) can provide a foundation for regulatory action and for the development of such non-animal methods by transparent and robust mapping of the available toxicological evidence and knowledge gaps. Furthermore, SRs can provide fundamental support for the development of alternative test methods that can efficiently assess chemical safety. The current toxicological SR therefore aims to characterize pollutant-induced cardiotoxicity while supporting regulation with a transparent and actionable overview of current knowledge and concerns. Using the NTP Health Assessment and Translation (HAT) approach for conducting toxicological SRs, we selected 360 references with *in vitro* (17%), *in vivo* (67%), and combined (16%) study setups for 129 potential cardiotoxic environmental chemicals, including heavy metals (29%), air pollutants (16%), pesticides (27%), and other chemicals (28%). Evidence maps and interactive knowledge graphs illustrate evidence streams, cardiotoxic effects and associated quality of evidence for chemicals, helping researchers and regulators to efficiently identify chemicals of interest. These results demonstrate toxicological evidence for pollutant-induced cardiotoxicity and highlight the importance of updating regulatory frameworks for human cardiotoxicity assessment. Furthermore, this SR substantiates the need for adequate chemical cardiotoxicity characterization and supports the development of the animal-free ALTERNATIVE test platform.

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Development of a physiologically-based kinetic model as part of a new approach methodology for non-animal cardiotoxicity testing

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The European Horizon 2020 ALTERNATIVE Project aims to develop an animal-free testing platform for assessing the cardiotoxic potential of environmental chemicals via a 3D cultured hiPSC-based microphysiological human heart model. By using physiologically-based kinetic (PBK) modelling, we aim to bridge the gap between *in vitro* toxicity estimates for a set of relevant environmental chemicals and *in vivo* human exposure estimates. This should facilitate subsequent risk assessment efforts. The classic cardiotoxic anthracycline doxorubicin (DOX) was tested in the ALTERNATIVE platform as proof of concept. In the microphysiological platform, DOX induced concentration and time-dependent increases in ROS while reducing cardiomyocyte viability, which is in line with evidence suggesting mitochondrial dysfunction is involved in DOX toxicity. To extrapolate these toxicological *in vitro* points of departure (PoD) to human dosimetry, we developed a mechanism-based full-body PBK model, which includes a detailed heart compartment consisting of epi-, mid- and endomyocardial sections. We included DNA, mtDNA, and cardiolipin binding processes in each section. To verify the model's performance, we simulated therapeutically relevant doses of DOX, which are known to lead to cardiotoxicity under clinical conditions. The resulting intracellular, extracellular, and total heart concentrations for these dosing scenarios corresponded to the ALTERNATIVE *in vitro* PoDs. This proof-of-concept study shows that *in vitro* derived concentration-response data can inform human dosimetry through the use of a PBK modelling approach. Our PBK model will be used in upcoming studies to simulate cardiac exposure to environmental chemicals tested in the ALTERNATIVE platform.

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Utility of developmental neurotoxicity *in vitro* battery to address regulatory challenges

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Limitations to the developmental neurotoxicity (DNT) test are well known, including high variability, low reproducibility, and limited historical control data. Neuropathological and neurobehavioral assessments in the DNT guideline are challenging due to methodology, lack of mechanistic understanding, and the high level of expertise required to conduct these assessments. The DNT *in vitro* battery (IVB) is an alternative testing strategy that is mechanistically informed and deemed ready for use in the regulatory arena. This case study investigates the utility of the DNT IVB to fill data gaps from an existing *in vivo* DNT study to refine toxicity endpoint selection for regulatory risk assessment. Acibenzolar-S-methyl was tested in the DNT IVB, and data were analyzed using the US EPA ToxCast Pipeline. Outcomes were examined in terms of

consistency of evidence between assays assessing the same neurodevelopmental endpoint and with known biological relationships. Results of the battery were compared to *in vivo* results using an Integrated Approaches to Testing and Assessment framework. Examination of outcomes across the DNT IVB revealed that acibenzolar-S-methyl does not interact with fundamental neurodevelopmental processes *in vitro*, such as proliferation, migration, apoptosis, differentiation, neurite maturation, synaptogenesis, and neural network connectivity. Integration of findings from the DNT IVB with *in vivo* data demonstrated no biological plausibility of a developmental neurotoxic mode of action as originally speculated from the *in vivo* results. This case study highlights the applicability of the DNT IVB in addressing data deficiencies and regulatory concerns of a DNT hazard without the need to conduct further vertebrate testing.

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Does good welfare make good science? How rodent housing impacts research results

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Over 120 million research rodents are used annually, most housed in small, barren "shoeboxes". Researchers' fears of altered results are one obstacle to improving these conditions. Yet if results do fail to generalise, this could help explain why even replicable studies (which comprise under 50% of publications) often fail to translate to humans. We hypothesized that the stressful nature of rodent housing reduces both data replicability and translatability, by generating animals that differ biologically between laboratories, and are too abnormal to model most humans. Our previous work demonstrated that compared to larger, more complex cages, conventional cages increase rodent susceptibility to stress-sensitive disease and reduces their lifespans. Here, we build on this by testing the critical prediction that statistical interactions between housing conditions and "disease modifiers" being studied (e.g., therapeutic drugs, sex or age) qualitatively alter results. We tested this via a systematic review and meta-analysis of studies examining effects of disease modifiers on stress-sensitive disease outcomes, in conventional cages and larger, well-resourced ones (n = 64). In two-thirds of these, housing and disease modifiers did not interact. Refining housing would thus not change research results in these cases. Interactions did occur in the remaining third of studies: here, rodent housing did change results. Housing can thus influence replicability (emphasising the importance of reporting cage conditions). Housing-stressed subjects can also generate different findings, questioning these altered results' relevance to humans, and suggesting that housing refinements would promote translatable science as well as improved animal welfare.

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An adverse outcome pathway for histone deacetylase inhibition leading to axial skeletal defects: Development and potential to improve decision support in chemical safety assessment

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Developmental and reproductive toxicity (DART) is an endpoint of regulatory significance. Application of adverse outcome pathways (AOPs) supports alternative testing approaches to safety assessments. The practical uses of AOPs include: 1) review of all relevant data for a specific compound, 2) prioritisation of assay selection given mechanistic hypotheses, and 3) broaden understanding of toxicity potential of a new substance through the review of data for similar compounds. Here we illustrate development of a new DART AOP, and its application in regulatory decision support. A new DART database, constructed based on studies according to the relevant guidelines (OECD TG422/TG421) under the Japan Chemical Substances Control Law, was curated and analyzed. A target was selected from the analysis and an AOP was constructed which focused on describing the main evidence that indicated a causal relationship between a target and an adverse outcome. The developed AOP, starting with histone deacetylase (HDAC) inhibition as the molecular initiating event, involves key events in three pathways (impairments in gastrulation/chondrogenesis/osteogenesis) leading to axial skeletal defects as the adverse outcome. The mechanism is well supported by observations from both knockout studies and toxicological studies involving structurally diverse HDAC inhibitors. Using valproic acid (and its analog) and a statin derivative as examples, the three regulatory applications of the AOP were successfully performed. Our work demonstrates how new AOPs and assay data associated to their key event can contribute to a comprehensive DART evaluation strategy.

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Building trust in new approach methodologies via the RE-Place project

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Even though most New Approach Methodologies (NAM) are very promising, their actual implementation, especially for regulatory applications, is lagging behind. Biomedical research is focused on the development, optimization and practical application of new methods and models, rather than fine-tuning protocols for future validation. Research groups may work in parallel, resulting in (slightly) different methods and models and thus leading to a lack of standardization, further hampering the regulatory acceptance.

In Belgium, the RE-Place project aims to tackle this challenge with a bottom-up strategy focused on promoting the use of NAMs and bridging the gap between method developers and their users. The major objective of this project is to map all available knowledge on NAMs in one central database. This open access database, available via www.RE-Place.be, provides an up-to-date overview of the different NAMs including the names of the experts using them in Belgium. By providing a direct point of contact, networking activities are greatly facilitated, especially between different stakeholders (scientists, ethical committees, regulators, …).

Almost 6 years after its launch in 2017, the database contains now more than 200 methods coming from various scientific areas. Convincing scientists to collaborate, remains the main challenge. Several concrete incentives for scientists to contribute were created (including poster awards and publications), but one of the most crucial elements was, and is, building trust by setting-up personal meetings. Trust is indeed, besides training and transferability, a key element which will help to exploit the full potential of NAMs and the RE-Place project.

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Building a sustainable compassion science program across multiple countries and cultures

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Multiple studies looking at the role of compassion fatigue and mental wellbeing in Lab Animal Professional have identified ways to address compassion stress and fatigue by developing strategies that support building resiliency and increasing compassion satisfaction. Developing sustainable programs that address individual employees' needs, respect site culture, and have the capcity to grow overtime is key to supporting an emotionally engaged workplace. To address the effects of compassion stress and fatigue and increase resiliency in a sustainable way, we developed and implemented a corporate program to meet the needs of personnel across multiple sites and geographies. We have utilized multi-tiered systems of supports to build collaborative relationships with management and those implementing the program locally. This includes ongoing communication efforts to raise the visibility of the program, training for management, supervisors, team leaders, and > 9000 of those working in the vivarium caring for the animals, webinars, newsletter articles, posters, and more. To build the program at the site level, we have more than 90 local Resiliency Building Ambassadors (RBAs) at 31 sites in the US, Canada, and EU. We are seeing continued interest in training opportunities and efforts to help support employee well-being across business units, geographic regions, and cultures. Through firsthand experiences, feedback from internal surveys of Frontline Leaders, RBAs, and employees that work with animals, participants will learn from the many challenges we have encountered and the valuable lessons we have learned throughout this process that can inform efforts to implement Compassion Science programs at their facilities.

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Non-animal antibodies in new approach methodologies implementation

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Strategies for generating non-animal-derived antibodies have been heavily explored in recent years, but they still deserve to be more widespread, evidencing their biomolecular relevance. Knowledge of the genomic sequence of non-animal antibodies or recombinant monoclonal antibodies (rMAb) provides a unique, versatile, sustainable, and renewable identifier, with the production of uniform batches in sufficient quantities to meet large demands and improve experimental reproducibility. Therefore, we seek to update the scientific community on the options, application possibilities and sources of supply of non-animal-derived antibodies worldwide to encourage their application, production, funding and ethical review. Despite advances in New Approach Methodologies (NAMs) for laboratory tests, many studies have not yet abolished the use of animal products, as is the case in the production of antibodies. As a scope review, a synthesis of the studies was conducted following a systematic approach to map the evidence on the subject and identify the main concepts, theories, sources, and knowledge gaps related to the use of rMAb. In this study, studies describing the use of rMAbs produced from hybridomas were carefully analyzed. Frequent systematic reviews on this subject are essential to support the development and adaptation of animal-free NAMs with the inclusion of rMAbs. Incentives and investments for the production of animal-free antibodies need to be motivated to warm up this innovative and economically favorable expanding market.

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Development of a new reconstituted human airway model to assess inhalation toxicity

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In 2011, polyhexamethylene guanidine phosphate (PHMG) was used as a disinfectant to prevent growth of microorganisms in humidifiers. However, exposure to PHMG was suspected as a causative agent of lung disease in Korea. Thus, determining the toxicity potential of compounds is of importance for health safety. Currently, there are no adequate predictive *in vitro* models available. Therefore, this study developed a novel *in vitro* three-dimensional human airway model (HAM model) to predict toxicity as an alternative to animal testing. Two of microbicides were treated to HAM model, and after three-hour incubation, the tissues were washed with DPBS. Test chemicals were removed, and values of cell viability, cytokine through ELISA and TEER were measured. By the results, we could predict that PHMG and bleomycin possessed irritant potential on the human airway model. Therefore, HAM model can be used as a good alternative test method for assessing toxicity of chemicals.

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A pilot study predicting animal-to-human translation using qualitative comparative analysis

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Background: Drug development is hindered by high attrition rates; promising candidate drugs fail in clinical trials. Attrition may result from low animal-to-human translation. Our previous study shows translational success rates ranging from 0% to 100%. That study could not predict translational success from individual factors.

Aim: Testing interactions between factors using two approaches: Qualitative comparative analysis (QCA) and regression analysis (RGA).

Approach: With RGA it is challenging to analyse multiple interactions and specific configurations (≈ combinations) of predictors, which is improved with QCA; an approach based on set the-

ory and Boolean algebra. We reanalysed our preceding review data with a pilot QCA and compared it with RGA. The following definitions were used: old: ≤ 1999; large: n ≥ 75, successful translation: > 85% correspondence. Included studies assessed one or multiple animal species, at either event/study or intervention level, and they defined translation quantitatively or qualitatively.

Results: This pilot QCA resulted in a preliminary formula for successful translation:

~Old*~Intervention*~Large*MultSpec*Quantitative

As ~ means not, the formula means that the combination of relative novelty, analyses of n < 75 at event or study level of multiple species with quantitative definitions resulted in successful translation. A separate QCA for translational failure will be shown in the presentation. When we analysed the same data with an RGA, none of the included factors showed significant contributions.

Conclusion: Because these data were not collected with a QCA in mind, the results should be interpreted with caution. We do however convincingly show that the QCA approach is viable in this context.

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Quantifying animal-to-human translation and comparing them across medical fields

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Background: Results from successful preclinical developmental experiments can often not be reproduced in clinical tests. This may partially be explained by poor animal-to-human translation. If quantitative translational success rates would vary substantially between medical research fields, detailed analyses of common practices in these fields can aid in the identification of factors contributing to successful translation.

Aim: Assessing translational success rates in various medical research fields using two methods.

Approach 1: Successful results from phase-2 clinical trials, retrieved from the WHO trial register, were used as a proxy (i.e., an indirect measure) for translational success. Trials were categorized according to the international classification of disease (ICD-10) to allow for comparisons between fields.

Approach 2: A scoping review of published translational success rates was performed for three fields: neuroscience, cancer, other pharmacology.

Findings: Fields with the highest clinical trial success rates were: disorders of lipoprotein metabolism (86.0%) and epilepsy (85.0%). Fields with the lowest clinical trial success rates were: schizophrenia (45.4%) and pancreatic cancer (46.0%). From the 117 review papers included into our scoping review, we deduced comparable translational success rates between the fields of pharmacology (72%), neuroscience (62%) and cancer research (69%).

Conclusion: Our analyses suggest relevant differences in success rates between medical research fields. Based on the clinical trials, comparisons of practice between, e.g., epilepsy and schizophrenia might identify factors which could influence translational success. Ideas on further studies of differences in research practice between the research fields, aiming to improve translational success, will be discussed.

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GARD™: A study to investigate the applicability domain for agrochemical formulations

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The Genomic Allergen Rapid Detection (GARD™) is a genomic-based assay platform, which addresses Key Event 3 (dendritic cell activation) of the skin sensitization Adverse Outcome Pathway. The purpose of this work is to verify the applicability domain of GARDskin and GARDpotency, for the product class of agrochemical formulations, providing confidence and facilitating future regulatory uptake in our industry sector.

Forty-two agrochemical formulations were tested using GARDskin. When GARDskin was positive, GARDpotency assay was used to determine potency (GHS category). The selected formulations were mostly liquids (15 water-based, 15 organic solvent-based; 12 solid) with a balanced distribution (23 not classified; 18 GHS cat 1B; 1 GHS cat 1A, rare for agrochemical formulations). Tests were conducted according to the assay developer Standard Operating Procedures and a nominal average molecular weight. GARD results were compared with already available *in vivo* data, in order to verify concordance (GHS hazard and potency categories). Initial results for the first 38 formulations, GARDskin was able to correctly identify 15/21 not classified (true negatives)

and 14/17 GHS1B/1A (true positives), with 3 false negative and 6 false positives. The accuracy, sensitivity, and specificity were 76.3% (29/38), 82.4% (14/17), 69.2% (9/13). GARDskin mispredictions were associated with borderline *in vivo* outcomes, and genomic signatures were assessed for causal relationships. Additionally, GARDpotency was able to correctly identify 9 GHS cat 1B out of 12 correctly predicted sensitizer (1 underprediction and 2 overprediction occurred).

In conclusion, GARDskin and GARDpotency, showed a satisfactory performance in this initial proof of concept.

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Skin sensitization and photosensitization evaluation through LC-MS/MS, HRMS and 3D reconstructed tissue approaches: An integrative evaluation strategy addressing the mixtures challenge

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Sensitization evaluation is an essential part of the safety assessment in the industry and to comply with the current ban of *in vivo* assays for the evaluation of cosmetics raw materials in Europe, several *in vitro* assays were developed for the evaluation of these substances without the use of animals.

In parallel, the skin sensitization process was conceptualized as an Adverse Outcome Pathway (AOP) based on four key events (KE), and the existing validated assays were linked to specific KE of the AOP through defined Integrated Approaches to Testing and Assessment (IATAs).

However, these tests were developed for simple substances evaluation and not for substances difficult to test such as lipophilic substances requiring the use of organic solvents, or complex mixtures such as botanical extracts. Although possible, the testing was shown to be difficult mainly due to solubilization issues, to interference related to cytotoxic effects from the organic solvent or from a component of the mixture, or to the unavailability of the mixture composition.

We discuss here the limits of the tests described in the standard guidelines, together with the options developed at Oroxcell to address these issues, both by setting up innovative mixture characterization methods, by the development of new assays and by the adaptation of existing tests to substances difficult to test. Our integrative approach considerably extends the field of application of the assays towards complex substances and mixtures for toxicological assessment and efficacy against sensitization effect, and for the evaluation of photosensitization effect of these substances.

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High-throughput toxicity screening and transcriptomic analysis in embryonic zebrafish as a NAM for PFAS assessment

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Per- and poly-fluoroalkyl substances (PFAS) are artificial chemicals that have been used in diverse industrial applications and commercial products. Although some PFAS have been phased out due to their adverse toxicological effects, over 9000 PFAS have been identified in humans and the environment. Given the globally expanding use of PFAS, new approach methodologies (NAMs) are needed to rapidly identify their potential for toxicity. Embryonic zebrafish less than 5 days post-fertilization (dpf) are an emerging 3Rs-compliant model that enables evaluation of systemic effects encompassing critical developmental stages. We are establishing this model in combination with advanced omics technologies to assess the toxicity of PFAS. Transcriptomic profiles on groups of untreated zebrafish were produced using S+1500 TempO-seq analysis to identify the optimal embryo pool size and conduct a power analysis. Zebrafish embryos staged 3-4 hours post fertilization were exposed to eight PFAS of differing chain lengths (i.e., PFDA, PFDS, PFOA, PFOS, PFHxA, PFHxS, PFBS, and PFBA), until 5 dpf. We evaluated developmental toxicity, behavioral changes, metabolic activity, and transcriptomic changes. Dose-response modeling of phenotypic and transcriptomic changes was performed with BMDExpress v2.3. The potency of the PFAS on the embryos was PFDA > PFDS > PFOS > PFHxS ≈ PFOA ≈ PFHxA > PFBS ≈ PFBA. These results are consistent with expectations based on chain length, supporting the suitability of our model and study design. Moving forward, this NAM will be used to establish points of departure for use for ecological and human hazard assessment.

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Use of the *in vitro* monocyte-activation test (MAT) for vaccine products that are inherently pyrogenic

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To ensure safety and to meet regulatory requirements, vaccine manufacturers must demonstrate that their products contain acceptable levels of pyrogens. Pyrogens, both endotoxin and non-endotoxins, are currently detected using rabbits inoculated with products in the "Rabbit Pyrogen Test" (RPT). To reduce or eliminate the use of these animals, Sanofi Vaccines has developed and qualified the Monocyte-Activation Test (MAT) for testing drug products for pyrogenicity. The MAT is an *in vitro* assay that detects the presence of pyrogens in a sample by incubating it with human monocytes. If pyrogens are present, the monocytes will be stimulated to produce cytokines. The measurement of cytokine response (in this case, IL-6) from a sample is compared to that of controls and used as a readout to assess the pyrogen content. The European Pharmacopoeia currently endorses three MAT Methods (A, B and C). Amongst these, Method C, comparing the response induced by a test lot with that of a reference lot, was identified as the most suitable for the testing of inherently pyrogenic products. Here, we report on the development and qualification of the MAT for one of Sanofi's products. In this context, specificity (including the detection of endotoxin and non-endotoxin pyrogen spikes), precision, and linearity were assessed. Following these evaluations, the assay was determined to be suitable for use. Implementing the MAT will allow for the monitoring for safety and consistency without the use of rabbits, which is in line the company's adherence to the 3Rs principle.

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Developing a triculture *in vitro* human gut model to evaluate methods for micro and nano plastics toxicity testing

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Micro and nano plastics (MNPs) are contaminants of increasing ecotoxicological concern in aquatic environments, as well as for human health. However, data regarding the risks of MNPs to humans are still limited and is needed for improved hazard assessment. Reliable methods are needed to evaluate the potential interactions between MNPs and the human intestinal barrier after ingestion. These measurements can be done using *in vitro* models. In this study, a 90/10 Caco-2/HT-29 and Raji-B co-culture system was set up using differentiated cells to mimic the human intestinal epithelium. Confocal laser microscopy was used to characterize the model after 21 days of co-culture. Furthermore, the *in vitro* model was exposed to polystyrene MNPs for 48 h to evaluate paracellular transport, trans-epithelial electrical resistance (TEER), and cell viability. MNPs were characterized using dynamic light scattering (DLS), field-flow fractionation, zeta-potential, and fluorescence lifetime imaging microscopy (FLIM). Each endpoint was evaluated to determine control measurements suitable for MNPs toxicity testing.

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Incorporating GIVIMP recommendations into method development, use, and transfer

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The demand for toxicology data from human relevant, New Approach Methodologies (NAMs) continues to increase. Fortunately, the scientific community has responded with new tools based on human tissues and cells. The creators of these systems and test methods utilizing them have invested in their development and also in studies designed to demonstrate their relevance, reproducibility and transferability. Despite these efforts, scientific confidence in these methods at the regulatory level remains compara-

tively low. In order to bridge the gap between development and acceptance, industry best quality practices need to be embraced by all stakeholders as early as possible in development and continue throughout the life cycle of a test method. The Good *In Vitro* Method Practices guidance document was published by the OECD in 2018 with an aim to "improve the reliability and robustness of *in vitro* methods, reducing the uncertainties of *in vitro* based predictions and therefore increasing the acceptance of the *in vitro* estimated safety measures by regulatory agencies" (OECD, 2018). The magnitude of the information presented in GIVIMP has led to challenges in its uptake and use by the *in vitro* testing community. This presentation provides points to consider for method developers and users, and test system providers implementing GIVIMP guidance within their laboratories as one step to improving scientific confidence in NAMs. Incorporating GIVIMP standards into standard laboratory procedures will improve the transparency and reproducibility of the methods developed and performed there, and increase the confidence of validation bodies, receiving authorities, and industry in NAMs.

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An integrative approach to develop NAMs for use in Alzheimer's disease research

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Alzheimer's disease (AD) is the most common form of dementia. The etiology of AD remains elusive, limiting therapeutic interventions to symptom management. For the last three decades, animal models have been used to elucidate the pathogenesis of AD and to test potential therapeutics. However, 99% of drugs that worked in animal models during the pre-clinical phases have failed in clinical trials. Therefore, it is critical to better understand the reasons for failure of animal models, the reticence observed in the adoption of NAMs, and barriers to designing more effective, human-relevant AD models. Interviews with AD researchers and literature review were used to understand the issues at play and delineate a path forward. The commonly used mouse models of AD require expression of chimeric and/or mutant human genes for AD pathogenesis. Most mouse models carry Familial AD mutations which account for less than 5% of all AD cases. Neurofibrillary tangles, commonly observed in AD patients, only develop in AD mice upon the expression of human tau containing mutations associated with frontotemporal dementia, reducing their clinical relevance and translatability. Non-human primate and canine models are cost-prohibitive, rarely form neurofibrillary tangles, and raise ethical concerns. More human-centered research includes brain-on-chip, organoids, epidemiological studies, and omics. However, these are not developed/validated enough due to limited funding. The adoption of an integrative approach where those developing/validating human-based, non-animal models, partner with animal researchers is recommended to better understand the knowledge and infrastructure gaps that exist in phasing out animal models in AD research.

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Analysis of pyrogen contamination using assay-ready THP-1 derived macrophages

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Testing for pyrogen contamination is one of the most critical QC assay for revealing regulatory approval since this type of contamination can cause severe reactions in the human body even on low concentrations. Therefor highly sensitive assays are needed to predict the pyrogen potential of a product. Commonly the rabbit pyrogen test is used to determine the risk potential but recently cell-based assay have also been accepted as alternative by pharmacopoeia, such as the MAT Assay. The monocyte activation test is capable of measuring the amount of pyrogen found in those products, it is therefore an efficient alternative to the undesirable rabbit pyrogen test.

We have tested THP-1 derived macrophages which were cryopreserved as assay ready cells, meaning prior cultivation is not necessary, these prequalified assay ready cells are more precise than a continuous culture due to lack of variances from cell handling and passaging. The Assay Ready Cells function like a reagent in the monocyte activation test and were neither less sensitive, nor did they display a higher sensitivity when treated with endotoxins and non-endotoxin pyrogens compared to cells from a continuous culture.

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Effects of different home cage enrichment on group housed male mice

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It is widely recommended to group-house male mice because they are "social animals", but mature male mice do not naturally share territories and aggression can be a serious welfare problem. These territorial, fighting behaviours may be exacerbated in current housing conditions which can create significant welfare costs and cumulative suffering.

Here we assess whether changes in enrichment methods within a standard caging system can reduce the competition for resources and reduce aggressive social interactions. We used 3 C57/BL6 mice per cage, housed from 6 weeks old in standard size cages with a) no enrichment except nesting material, b) standard enrichment- single cardboard nest box, nesting material, chew block c) premium housing- two nesting areas, cardboard tube, chew block. We studied how these different forms of environmental enrichment affect measures of animal welfare including home-cage behaviour, reward and novelty induced neophagia.

Results show a lack of enrichment significantly increased anxiety in novelty suppressed feeding and there was an increased tendency towards fighting. In the premium housing there was a tendency for less fighting. However, considerably more premium enrichment groups were lost due to reaching endpoint from the severity of fighting. Our results highlight the difficulty in making general recommendations for enrichment strategies with both no enrichment and premium enrichment having potential welfare issues. Experiments of this nature would benefit from a considerable larger n number with observational data at an institutional level.

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A standardized score sheet template for assessing rodent health

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Score sheets formalize and standardize the assessment of welfare and make it possible to record the impacts of scientific procedures. The Swedish National Committee for the Protection of Animals used for Scientific Purposes and its group of experts has worked out a score sheet template for assessment of rodent health. The template is based on existing score sheets and further developed with advice from veterinarians from Swedish research institutions. The score sheet was published by the Swedish 3Rs Center for the purpose of national standardization.

The template can be used to assess animals' health before, during and after a study to estimate if the animals' health has deteriorated, as well as to decide if the humane endpoint is reached. It can be used by the principal investigator to set the humane endpoint in a study. The template lists several categories of factors to assess, including general condition, condition of the fur, skin and teeth and breathing. Each parameter is scored 0: no change, 1: slight change or 4: substantial change from normal. If the criteria for the humane endpoint is reached, the experiment should be stopped and the animal treated or euthanized, regardless of whether the purpose of the study has been achieved or not. Our template has been shared nationally and several facilities have started to use it to assess the health of their rodents.

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Swedish acclimatization guidelines for rats and mice

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Acclimatization after transport is commonly used to reduce stress after, e.g., transport to a new facility, and required in the Directive 2010/63/EU. However, details on how to acclimatize animals are not provided and there is a need for harmonized guidelines to improve scientific comparison. As a first step towards formulating guidelines for acclimatization of rats and mice, the Swedish 3Rs Center, together with veterinary students, sent out an anonymous survey to Swedish research facilities, investigating how, when and for how long they acclimatize their rats and mice.

Preliminary results indicate large differences among Swedish research facilities. Most respondents (91%) answered that rats and mice are acclimatized after transportation to the facility. However, the acclimatization period ranged anywhere between 3 days to 4 weeks, with most respondents indicating 5-7 days or around two weeks. The majority (53%) answered that there are written instructions for acclimatization within their organization, the rest answered no (13%) or do not know (34%). When asked if animals are acclimatized after other changes to their daily routines, most respondents answered that they do not acclimatize or do not know if acclimatization occurs when animals are being moved within the facility (75%), experiencing changes to the circadian rhythm (80%) or being regrouped (86%).

The survey shows that guidelines are needed to harmonize acclimatization of research animals in Sweden. Together with experts, we will compile scientific evidence with the survey results, to create a guide of best practice on acclimatization of mice and rats in experiments.

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Guidance for reviewing projects involving the use of the forced swim test

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The forced swim test (FST) was once among the most widely used tests for screening antidepressant drugs, and it is also used in an attempt to model human depression in animals. Now, universities, pharmaceutical companies, and regulators around the world are discouraging use of this procedure because of increasing evidence that it is a poor model of depression, is unreliable at predicting antidepressant efficacy, and could even hinder the development of effective new treatments. A literature review of articles published in the UK and EU in 2022 assessed the scientific rationale provided for using the FST and any commentary included on the ethical approval of the procedure. The review identified 49 publications that used the procedure – 47 of them in the context of depression research. To publish research using animals, a project may undergo review at several stages, including by funders, local and national committees, and journals. Aspects reviewed may include a project's scientific quality, adherence to the 3Rs, and whether the harms caused to animals can be considered justified by the scientific gains of the project. A large number of studies continue to be approved and published in which the FST is used in an attempt to understand or find treatments for human depression. We summarise the scientific and welfare concerns of the FST, make recommendations to support those involved in the project review and authorisation process, and provide valuable information about the procedure for funders and journals.

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Plant extracts, polymers and new approach methods: Practical experience with skin sensitization assessment

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Over the last decade, research into methodologies to identify skin sensitization hazards has led to the adoption of several non-animal methods as OECD guidelines. However, predictive accuracy beyond the chemical domains of the individual validation studies remains largely untested. In the present study, skin sensitization test results from *in vitro* and *in chemico* methods for 12 plant extracts and 15 polymeric materials are reported and compared to available *in vivo* skin sensitization data. Eight plant extracts were tested in the DPRA and h-CLAT, with the 2 out of 3 approach resulting in a balanced accuracy of 50%. The balanced accuracy for the 11 plant extracts assessed in the SENS-IS was 88%. Excluding 5 polymers inconclusive *in vitro*, the remainder, assessed using the 2 out of 3 approach, resulted in 63% balanced accuracy. The SENS-IS method, excluding one polymeric material due to technical inapplicability, showed 68% balanced accuracy. Although based on limited numbers, the results presented here indicate that some substance subgroups may not be in the applicability domains of the method used and careful analysis is required before positive or negative results can be accepted.

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Thyroid organoid-on-a-chip batteries for screening endocrine disruption

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Exposure to Endocrine Disrupting Chemicals (EDCs) has been associated to thyroid malfunctioning in the human body. Despite their ever-increasing impact on human health and well-being, the identification of EDCs is still challenging due to the lack of representative thyroid toxicological assays. New and more advanced thyroid models are urgently needed to better mimic the thyroid gland and accurately predict EDC responses *in vitro*. Here, we developed a microphysiological flow battery capable of screening the effects of multiple EDCs on complex 3D thyroid organoids. Each microphysiological flow battery was composed of six reversibly sealed bioreactors that allowed quick and efficient (un-)loading of mouse or human embryonic stem cell (ESCs)-derived thyroid organoids. Under peristaltic flow, thyroid organoids were capable of recapitulating the follicular 3D architecture of the native thyroid gland with expression of key thyroid genes and with accumulation of T4 hormone in the luminal space. Finally, the microphysiological batteries were exposed to sixteen different EDCs and molecular changes were evaluated using downstream transcriptomic and proteomic analysis. Upon EDC exposure, thyroid follicles revealed changes in gene and protein expression and new modes of action were investigated. Altogether, we believe that our platform brings valuable insights on the development of next-generation preclinical toxicological assays to potentially replace or reduce the use of animal models in early detection of EDCs.

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Fever pitch: Restoring global harmonization of bacterial endotoxins testing

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Globally, every lot of parenteral drugs, vaccines, and medical devices must be tested for pyrogenic contamination. The most common pyrogens, bacterial endotoxins, can be detected *in vitro* using lysate of the hemolymph of the horseshoe crab, an extraordinarily unique arthropod with limited coastal habitats. Within its blood, the horseshoe crab produces a clotting cascade that is highly sensitive to endotoxins. However, fully functional clotting factors can be produced via recombinant protein production, eliminating the need for traumatic collection and bleeding of Endangered and Vulnerable species to ensure vaccine and biologics batch safety. As part of efforts to fully phase out animal use in pyrogenicity testing, a new European Pharmacopoeial chapter was approved in 2020. Ph. Eur. Chapter 2.6.32 covers the use of Recombinant Factor C for Bacterial Endotoxins Testing (BET). However, the United States Pharmacopoeia (USP) has not yet approved an analogous compendial chapter for recombinant methods, citing concerns about data for comparison. Two roundtables were convened in 2021 to bring pharmaceutical stakeholders, regulatory agencies, and test method developers together to discuss barriers to the use of recombinant BET methods in the US. We found that there was consensus that recombinant Factor C methods have been shown to meet performance criteria for analytical procedures as described by both the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 and USP <1225>. Most stakeholders agreed that a compendial US chapter should be established without delay to help restore global harmonization of endotoxins testing.

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Galleria mellonella as an alternative model to study biodefense pathogens

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The 3Rs principle of Russell and Burch in 1959 instigated a search for alternative animal models in scientific research. This has become one of the most important goals underpinning research today due to ethical, legal, and economic drivers to replace, reduce, or refine the use of regulated mammalian animal species. One such alternative model is the invertebrate Greater wax moth larvae, *Galleria mellonella*, that has been used as an alternative infection model for bacterial and fungi pathogens of humans since the 1980s. There are several advantages of using *G. mellonella* since they can be maintained at mammalian body temperature, are cheap and simple to manipulate. They also possess several functional homologues of components of the innate immune response of mammals.

We have evaluated *G. mellonella* as an alternative model to study infections caused by potential biothreat pathogens such as *Francisella tularensis*, *Burkholderia pseudomallei*, *Coxiella burnetii*, *Yersinia pestis*, Ebola virus and Venezuelan Equine Encephalitis virus. We have also evaluated *G. mellonella* as a model to determine the toxicity and pharmacokinetic parameters for a number of classes of clinically relevant antimicrobials and have used this model to assess the efficacy of a panel of novel antimicrobials. A range of metrics have been used to monitor infection, including survival, bacteriology/virology and imaging techniques. These results demonstrate the utility of the *G. mellonella* as an alternative model to replace and reduce the use of mammalian models in biodefense research.

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Enhancing the E-Morph assay for phenotypic screening of endocrine disrupting chemicals

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With an all-time high of over 2 Mio diagnosed cases, breast cancer is the most frequently diagnosed cancer type and one of the leading causes of death in women worldwide. The disruption of physiological estrogen function by environmental chemicals is one of the factors that potentially contribute to breast cancer progression. Thus, identification of substances that interfere with hormone pathways and elicit adverse effects, so-called endocrine disrupting chemicals (EDC), is of high relevance. We developed the E-Morph Assay (Kornhuber et al., 2021; Klutzny et al., 2022), which quantifies estrogen-dependent phenotypic reorganization of adherens junctions in MCF-7/vBOS cells to identify and characterize estrogenic and anti-estrogenic hormone activities of test chemicals us-

ing a functional and potentially clinically relevant endpoint.

In order to expand the E-Morph Assay's screening capability and human-relevance, we up-scaled its high-throughput screening (HTS)-testing capacity through further automation and miniaturisation and adapted MCF-7/vBOS cells to defined, serum-free cell culture conditions. To further characterize the molecular mode-of-action underlying the estrogen-dependent phenotypic readout of the E-Morph Assay and to identify other hormonal pathways leading to similar morphological phenotypes, we have screened the EU-OPENSCREEN Bioactives Library. Changes in cellular architecture upon exposure to the 2,500 reference compounds with known bioactivity are currently investigated in detail using the CellPainting method.

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Teratogenicity assessment of antimalarials early in drug development using the ReproTracker assay

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Malaria is a widespread disease affecting millions of people annually, especially pregnant women. Over the past decades, several novel antimalarials have been developed. Unfortunately, many of these have been shown to be developmental toxicants (teratogenic). This highlights the need for continued improvement and development of novel antimalarials with a low probability of developmental toxicity.

Currently, the assessment of potential developmental toxicants relies mainly on mammalian-based models, which do not always recapitulate the human condition and are run very late in the drug development process. To assess the teratogenic potential of antimalarials and to de-risk these chemicals as early as possible, we selected compounds where the data from mammals (embryofetal developmental studies) was already available and tested in the human stem cell-based test, ReproTracker. The ReproTracker assay is a state-of-the-art human induced pluripotent stem cell-based biomarker assay that can identify the teratogenic potential of new chemicals with high accuracy (86%), sensitivity (86%), and specificity (86%).

Overall, this comparative study has demonstrated that the ReproTracker assay is a valuable tool to improve the *in vitro* safety evaluation of novel drug candidates in an early stage of drug development. Furthermore, the study aimed to bring forward a broader normative framework for the implementation of preclinical models in development and reproductive toxicology studies (DART) and make explicit possible normative presuppositions behind the current practice of (the lack of) using animal-free models in DART studies.

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Reconstructed human epidermis: IVPT to compare topical products

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Interchangeable medicines known as generics refer to drug products that are pharmaceutically equivalent and bioequivalent to innovator products. Depending on the country, the evaluation of the bioequivalence of topical dermatological products follows different requirements and regulations. In this way, the use of *in vitro* human skin permeation tests (IVPT) has been of value when addressing the quality and equivalence of topical drug products. Considering that reconstructed human epidermis (RhE) is a model validated as skin surrogates for safety tests, the main aim of this research was to use SkinEthic RHE/Episkin® by IVPT technique for the assessment of "sameness" of different terbinafine hydrochloride 1% cream. For IVPT test, the inserts with the skin were placed in a 12-well plate with 2 mL receptor liquid (LR) PBS + Tween 80 0.8% and kept in the incubator under agitation (250 rpm) throughout the study (48 hours) at 37 ±1°C, 5% CO_2 and ≥ 90% relative humidity. At each defined time point, 500 µL of LR were sampling. ~10 mg of each product was applied to the membrane (0.5 cm^2). Although the formulations have similar organoleptic characteristics, pH, content, spread ability and viscosity, the IVPT results showed a statistically significant difference between them (unpaired Student t-test, $p < 0.05$. Flux, permeability coefficient and retention in the skin corroborate with IVRT study. Despite the RhE models showing to be twice more permeable than human skin, these results corroborate the acceptance of RhE as biological membranes for IVPT in topical products comparison and discrimination.

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Defined approaches for EPA categorization assessing eye irritation potential of agrochemical formulations

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Many sectors have seen a complete replacement of the *in vivo* rabbit eye test with robust *in vitro* and *ex vivo* methods to assess the eye irritation potential of chemicals. Compared to the traditional *in vivo* rabbit eye irritation test, these methods offer increased reproducibility and equivalent or greater relevance to mechanisms associated with human eye irritation. While the use of *in vitro* and *ex vivo* methods to assess conventional agrochemical formulations is accepted by several regulatory agencies, the *in vivo* test remains the requirement for the United States Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP). Reliance on the *in vivo* rabbit eye test has continued, despite its demonstrated lack of reproducibility and relevance to humans, because the accuracy of data from new methods is determined solely by direct comparison to the results from the *in vivo* test. In this poster, we present the results from testing twenty-nine agrochemical formulations in the reconstructed human corneal epithelium test method, EpiOcular and the Bovine Corneal Opacity and Permeability (BCOP) assay with histopathology. These methods were selected based on their relevance to mechanisms of human eye irritation, their capacity to assess a broad range of severity, a broad applicability domain, and their inclusion in internationally adopted test guidelines. We present two defined approaches developed using EpiOcular and BCOP. Based on their demonstrated performance, these approaches are proposed to replace the *in vivo* test for assessing eye irritation potential of agrochemical formulations.

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Validation of the electrophilic allergen screening assay (EASA)

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The electrophilic allergen screening assay (EASA) is an *in chemico* assay to assess skin sensitization potential. The EASA uses nitrobenzenethiol (NBT) or pyridoxylamine (PDA) probes as surrogates for thiol- or amine-based proteins to mimic chemical binding to proteins, the initial key event in the adverse outcome pathway for skin sensitization. Probe depletion is measured by absorbance or fluorescence. A test substance is positive when it meets the positive depletion criterion for either NBT or PDA but negative when the depletion fails to meet the positive criterion for both probes. The U.S. Consumer Product Safety Commission (CPSC) and the National Institute of Standards and Technology (NIST) modified the original cuvette-based EASA into a 96-well format. Four laboratories participated in a validation study of the 96-well test: the Food and Drug Administration Center for Devices and Radiological Health, the U.S. Department of Defense Public Health Center-Aberdeen, Burleson Research Technologies, and CPSC/NIST (lead laboratory). The laboratories tested 20 coded reference chemicals from the OECD performance standards for the direct peptide reactivity assay and amino acid derivative reactivity assay test methods. Of these, 12 chemicals were tested three times to evaluate intralaboratory reproducibility. Performance of the EASA was evaluated by comparison with local lymph node assay outcomes. The results suggest that the EASA may be a useful non-animal alternative to identify potential skin sensitizers.

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Evaluation of skin sensitization classification rules to reflect human potency

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Approaches currently used to subcategorize skin sensitizers into GHS subcategory 1A ("strong," using an induction dose per skin area [DSA] of 500 µg/cm^2 or less) or 1B ("other than strong," using an induction DSA greater than 500 µg/cm^2) consider only the dose inducing the skin sensitization response and not the frequency of induced sensitization in human subjects. To address this limitation, we used a data set developed to support OECD Guideline 497 to conceptualize approaches that incorporate the number of sensitized subjects, such as the DSA at which one subject is sensitized (DSA1+) or the DSA at which 5% of subjects are sensitized (DSA05). Of the 605 test results in which the test substance was active, the DSA1+ subcategorized 209 test results in GHS 1A (DSA and DSA05: 59 and 184, respectively). For substances with multiple test results, reproducibility was approximately 90% for binary classification and 80-85% for GHS subcategorization when applying a standardized weight of evidence (WoE) approach. HPPT concordance with LLNA was 44% (46/55) for binary classifications and 61-63% (28-29/46) for GHS subcategorization. Both data types, however, showed very good (91-93%, binary) or decent (76-95%, subcategory) concordance when compared to an overall WoE consensus classification. This approach to classifying and subcategorizing sensitizers improves the prediction of potency while providing good reproducibility and concordance with animal reference data.

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Combining *in silico* and *in vitro* tools for assessing inhalation hazard of sodium dodecyl sulphate aerosols

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Lung surfactant is a complex mixture of surface-active phospholipids and associated proteins present as a thin film at the air-liquid interface of the fluid lining the alveolar surface. These components enable effortless breathing by lowering the surface tension to near zero values to prevent alveolar collapse at the end of expiration. Inhaled chemicals have the potential to interact with lung surfactant and inhibit its function. We investigate, using the constrained drop surfactometer, the effect of sodium dodecyl sulphate (a surfactant used safely in consumer products), on lung surfactant inhibition. This cell-free model has previously been used to assess chemicals where lung surfactant function inhibition was determined by using a minimum surface tension of 10 mN/m as the pre-determined cut-off point above which the alveoli in the intact lungs will collapse. To refine the determination criteria, we have used a mathematical model developed to measure changes in the viscoelasticity of the surfactant and to quantify the effect of SDS on surfactant rheology; the Fourier Mode Dynamic Tensiometry Method. Inhibition was correlated to the rate of exposure, i.e., the concentration of SDS multiplied by the infusion rate. This novel method of assessing the effect of aerosols on lung surfactant function and rheology could provide additional information for consumer safety risk assessments when combined with information from the toolbox of non-animal approaches currently available.

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Closing the gap for T cell-based skin sensitizer prediction

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Purpose: We aim to address the lack of new approach methodologies (NAMs) for chemical-specific T cell activation, which constitutes key event 4 in the adverse outcome pathway of skin sensitization.

Study design: Activation-induced marker (AIM) T cell assays and T cell receptor (TCR) sequencing were employed to characterize chemical-specific CD154+CD4+ and CD137+CD8+ T cells in human PBMC.

Results: We tested 2-fold serial dilutions of sensitizing chemicals in the AIM T cell assays to determine concentrations compatible with spectral flow cytometry and T cell function. Using optimal conditions, we then quantified chemical-specific T cells. This revealed unusually high frequencies for several metal (Ni^{2+}, Co^{2+}, Pd^{2+}) and non-metal allergens (e.g., 2,4,6-trinitrobenezenesulfonic acid, Bandrowski's base). For instance, we observed 0.3% (mean) Ni^{2+}-specific CD154+CD4+ T cells in response to 400 µM $NiSO_4$ in non-allergic individuals. The strong T cell activation was mediated by interactions reminiscent of superantigens. TCR binding points included distinct gene segments or single amino acids in the highly diverse main antigen-binding region (CDR3) such as a histidine for metal ions. Intra-individual TCR repertoire analysis captured extensive cross-reactivity among metal-specific TCRs while their local skin enrichment proves an involvement in human allergic contact dermatitis.

Conclusion: A strategy is presented to characterize and quantify chemical-specific T cells fast, sensitive and comprehensively, thus overcoming the limitations of proliferation-based or cytokine secretion assays. Unusually strong T cell activation may contribute to sensitizing potencies, and, together with cross-reactivity analysis, should be addressed to solve discrepancies and provide missing data in the risk assessment of sensitizing chemicals.

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A human thyroid-on-a-chip to test thyroid disruption

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Thyroid functionality can be disrupted by exposure to endocrine disrupting chemicals (EDCs), which may cause serious health problems. Current toxicological tests, using 2D cell culture or animal models, often fail to predict the safety of potential EDCs. In order to improve the safety evaluation of these chemicals, there is an urgent need for human *in vitro* models that better mimic thyroid architecture and function. Here, we combine 3D organoid technology and microfluidics to develop a human thyroid-on-a-chip. Human thyroid organoids were generated from embryonic stem cells. Thyroid follicles, the functional units of the thyroid gland, were then incorporated in a microfluidic device and cultured under flow conditions for 7 days. The expression of key thyroid genes was maintained inside the chip, and interestingly, follicles formed into large multi-luminal structures, which resembles the architecture of the native thyroid gland. Exposure to 10 µM benzo[k]fluoranthene (BKF) for 7 days induced changes in expression of thyroid genes as well as alterations related to activation of the xenobiotic aryl hydrocarbon receptor (AhR) pathway, as demonstrated by RNA sequencing. Altogether, we present a novel microphysiological human thyroid-on-a-chip model, which may advance the development of better predictive *in vitro* assays to identify EDCs to eventually reduce the use of animal models for this purpose.

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Web application to predict skin sensitization using defined approaches

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Defined approaches (DAs) combine data from specific information sources to predict toxicity. While certain DAs are accepted to identify potential skin sensitizers, the data interpretation procedures they use vary in logical complexity and can be time-consuming to apply manually. We have developed an open-source web application, the DASS App, to facilitate use of three DAs for skin sensitization (DASS). The app implements two validated DAs, known as the Two-out-of-Three (2o3) and the Integrated Testing Strategy (ITS), that are described in OECD Guideline 497. It also implements the Key Event 3/1 Sequential Testing Strategy (KE 3/1 STS) accepted by the U.S. Environmental Protection Agency. The three DAs available in the DASS App integrate data from three *in vitro* assays that represent three key events within the skin sensitization adverse outcome pathway: the direct peptide reactivity assay (DPRA), human cell line activation test (h-CLAT), and the KeratinoSens assay. To predict skin sensitization hazard, the 2o3 uses the consensus hazard across the three *in vitro* assays; ITS uses a scoring method with outcomes from h-CLAT, DPRA, and *in silico* predictions; and KE 3/1 STS sequentially evaluates outcomes from h-CLAT and DPRA. ITS can also be used to predict potency categorization. The DASS App enables users to computationally implement non-animal approaches to evaluate chemical skin sensitization without the need for additional software or computational expertise. The DASS App is available on the National Toxicology Program website at https://ntp.niehs.nih.gov/go/40498.

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Using NAMs to address variability and susceptibility across populations

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Humans typically exhibit different levels of susceptibility to toxic effects from chemical exposure. These differences result in population-level variations in chemical effects that in turn affect characterization of chemical risk. Quantitative evaluations of human population variability and susceptibility could provide more robust chemical risk assessments that are protective of all populations, especially those disproportionately affected by chemical exposures. Human cell-based and computational new approach methodologies (NAMs) have the potential to model human responses better than traditional animal tests and could be used to characterize variability and susceptibility of human populations. In October 2022, NICEATM hosted a symposium aimed at initiating conversations among government, scientific experts on NAMs, and the environmental justice community about the challenges associated with population variability and susceptibility in the context of chemical risk assessment. Speakers at the symposium presented case studies in which NAMs have been applied to characterize susceptibility factors such as life stage, genetics, or lifestyle. A panel discussion considered the challenges of developing NAMs that broadly represent population variability and highlighted the need to delineate differences in susceptibility due to genetic and other intrinsic characteristics versus those due to systemic social constructs. Breakout groups discussions addressed confidence in models of population variability and susceptibility, barriers to and opportunities for applying such models, and how to best engage with local communities. This presentation summarizes the key topics and discussion points from the symposium to define the state of the science.

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Pump-less, recirculating platform enables unidirectional perfusion of 3D printed tubular tissues

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The direction and pattern of fluid flow affect vascular structure and function, in which vessel-lining endothelial cells exhibit variable cellular morphologies and vessel remodeling by mechanosensing. To recapitulate this microenvironment, some approaches have been reported to successfully apply unidirectional flow on endothelial cells in organ-on-a-chip systems. However, these platforms either require bulky pumps or are limited to 2D monolayer cell cultures. Herein, we demonstrate a pumpless recirculating platform with an open-well design (UniPlate) that enables unidirectional media recirculation through 3D printed tubular tissue constructs on a programmable rocker. To ensure that the platform is scalable for industrial manufacturing, the device was made of polystyrene via injection molding in combination with 3D printed gelatin that serves as a sacrificial template. We first engineered tubular blood vessels with unidirectional perfusion. Then we expanded the design to incorporate duo-recirculating flow for culturing vascularized renal proximal tubules with glucose reabsorption function. In addition to media recirculation, human monocyte recirculation in engineered blood vessels was also demonstrated for over 24 h, with minimal loss of cells, cell viability, and inflammatory activation. UniPlate can be a valuable tool to more precisely control the cellular microenvironment of organ-on-a-chip systems for drug discovery.

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A pragmatic framework for the application of new approach methodologies in One Health toxicological risk assessment

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Globally, industries and regulatory authorities are faced with an urgent need to assess the potential adverse effects of chemicals more efficiently by embracing new approach methodologies (NAMs). NAMs include cell and tissue methods (*in vitro*), structure-based/toxicokinetic models (*in silico*), methods that assess toxicant interactions with biological macromolecules (*in chemico*), and alternative models. Increasing knowledge on chemical toxicokinetics and toxicodynamics (what the chemicals do with the body) obtained from *in silico* and *in vitro* systems continues to provide opportunities for modernizing chemical risk assessments. However, directly leveraging *in vitro* and *in silico* data for derivation of human health-based reference values has not received regulatory acceptance due to uncertainties in extrapolating NAM results to human populations, including metabolism, complex biological pathways, multiple exposures, interindividual susceptibility and vulnerable populations. The goal is to provide a standardized framework that applies integrated approaches with a focus on quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) to relate *in vitro* cellular exposures to human equivalent doses from which human reference values can be derived. The proposed framework intends to systematically account for the complexities in data interpretation to support human health safety decisions in diverse industrial sectors. Case studies of chemical entities, using new and existing data, are presented to demonstrate the utility of the proposed framework while highlighting potential sources of human population bias, and the importance of Good Method and Reporting Practices and of the use of Artificial Intelligence (AI) to accelerate regulatory life science process.

Reference

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Target group specific efforts to facilitate replacement of animal experimentation

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The Swedish 3Rs Center and its steering group, the Swedish National Committee for the Protection of Animals Used for Scientific Purposes, work with all three Rs. This abstract describes our work focusing on Replace and how our work is adapted to dedicated target groups.

We aim to increase knowledge about science and research methods already at an early age, to promote neutral discussion without polarization. Every year we meet students at a national science festival to discuss research and choice of methods. To this end, we have also compiled a digital education material about research, animal experimentation, ethics and animal-free methods.

We aim to increase collaboration and dialogue between government agencies and other stakeholders to, amongst other things, speed up development and validation of animal-free test methods. Each government agency that comes into contact with animal experimentation, directly or indirectly, has its own specific needs to move towards animal-free methods. Therefore, we also provide agency-specific education to increase knowledge about specific methods or concepts.

To facilitate networking and increase knowledge about animal-free methods, we have instated the Swedish 3Rs Network for Replace. The network has more than 300 members including researchers and representatives of government agencies, animal welfare bodies, research-funding bodies etc., and thus converge on several target groups.

The work to Replace animal experimentation is multifaceted and spans over divergent target groups. It is important to have dialogue with and involve the target groups to ensure that activities and efforts are relevant and useful.

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Selection of ligands using an *in vitro* platform to support vaccine antigen characterization

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To develop and license vaccines, it is critical to be able to identify, quantify, and analyze the safety, conformation, functionality, antigenicity and potency of the vaccine antigens. Animal-derived antibodies have been widely used in *in vitro* assays for these purposes, but they have several drawbacks, including instability or high production costs, not to mention the use of animals. Non-animal-derived antibodies and non-antibody affinity reagents are alternatives which offer significant benefits including shorter production time, higher selectivity and better suitability for non-immunogenic or toxic targets, all without the use of animals.

We evaluated different platforms that offer an alternative to animal-derived antibodies. Among these, a platform based on a synthetic Fab library associated with selection by phage display demonstrated its ability to generate, select and produce ligands with the expected attributes throughout different approaches. In this communication, we will outline 2 distinct examples.

We have implemented a strategy using this platform to select optimal ligands to develop subtype-specific and sustainable antigenicity assays for viral vaccines.

These projects demonstrate the suitability of the selected technology for generating alternative ligands that can be used in complex *in vitro* assays. Rapid screening and generation of candidate ligands with high selectivity are key benefits of the Fab phage display technology compared to the classic method of generating animal-derived monoclonal antibodies.

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Using ontologies to organize textual resources in life sciences for their integration into *in silico* and *in vitro* science

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Ontologies are used to support access to a multitude of databases that cover domains relevant information. Heterogeneity and different semantics can be accessed by using structured texts and descriptions in a hierarchical concept definition. We are interested in Life Sciences (LS) related ontologies including components taken from molecular biology, bioinformatics, physics, chemistry, medicine and other related areas. An Ontology comprises: (i) term connections, (ii) the identification of core concepts, (iii) data management, (iv) knowledge classification and integration to collect key information. An ontology may be very useful in navigating through LS terms. We want to explore with our collaboration partners and stakeholder networks the applied use of the available biomedical ontologies and frameworks, compare them and show their role in improving and accelerating their integration into *in silico* and *in vitro* life science.

References

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Variability of *in vivo* toxicology studies: Impact on NAMs

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Test method reproducibility is critical for producing data that can be replicated, easily interpreted, and used for actionable outcomes. Guideline *in vivo* toxicology studies have long been the default for chemical safety assessments for regulatory decision-making and thus are the standard against which new approach methodologies (NAMs) are evaluated. However, retrospective analyses have demonstrated substantive variability in data from these studies. This variability, which has a variety of potential sources, can confound the use of these *in vivo* studies as reference data for establishing confidence in NAMs. With interest increasing in integrating and implementing NAMs into regulatory decision making, it becomes imperative to understand the variability of reference animal data and how that may affect the NAM evaluation process. This presentation will describe variability evaluations conducted on several different standardized *in vivo* toxicology test methods, including both single and repeat-dose study designs. These evaluations have shown that independent replicates of these studies can have less than 50% likelihood of yielding the same hazard classification, particularly when the original test characterizes the substance as having a mild to moderate effect. We have compiled results from variability analyses, systematic reviews, and meta-analyses of *in vivo* toxicological studies to characterize sources of variability across various study types. An improved characterization of *in vivo* variability will support a better understanding of how to set appropriate expectations when building confidence in the use and interpretation of NAMs.

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Proficiency testing of *in vitro* methods

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The Brazilian Alternative Methods Network (ReNaMA) was created in 2012 by the Ministry of Science, Technology and Innovations and is divided into two types of laboratories: Central and Associated. The network currently has nearly 50 members from the public and private sectors. The central laboratory Inmetro (National Institute of Metrology, Quality and Technology) has organized interlaboratory comparisons to promote the continuous improvement of the network, using the standard ISO 17043:2010 (Conformity assessment – General requirements for proficiency testing) as a reference. Four interlaboratory comparisons, with 17 participants, were conducted between 2017 and 2019, focusing on OECD *in vitro* methods. The "value" of the test items came from the expected GHS classification. Regarding the correct GHS classification of the test items, 12 participants were successful. However, when we break down this information, we find that only one participant met the quality criteria outlined in the appropriate test method guideline. Recognizing that it was a first exercise, each participant also received feedback on technical issues that could be improved. Our results strongly suggest that periodic evaluation of laboratory performance by a third party would be helpful to improve the quality of the results, even though this is not required under the Good Laboratory Practice (GLP) management system. Given the increasing complexity of new technologies in this field, successful participation in interlaboratory comparisons, either under a proficiency testing programme or an external quality assessment (EQA), can be recognized by stakeholders, such as regulators, as evidence of the laboratory's quality and competence.

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Implementation of alternative methods in Argentina to evaluate skin and ocular irritation of cosmetics products

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The implementation of alternative methods in Argentina is a new challenge. Although there are currently no laws that restrict or prohibit the use of animals for the evaluation of cosmetic products in Argentina, due to ethical issues, social demand and to keep up with global advances, it is necessary to work on the implementation of methodologies without animals.

For this reason, Laboratorios Bagó SA decided to bet on the implementation of *in vitro* tests. It was decided to start with two tests to evaluate ocular irritation potential: STE (Short Time Exposure – OECD TG 491) and HET-CAM (hen's egg chorioallantoic membrane test); and an assay to assess skin irritation: SkinEthic™ OECD Test Guideline 439.

In a first stage, proficiency tests were carried out with chemical products recommended by the guidelines. Once methodological competence had been demonstrated, a series of test were carried out. We evaluate 48 products by HET-CAM, 15 products by STE and 8 products by SkinEthic. Among the product tested were antiseptic and healing creams; spray, cream and emulsion sunscreens; hydrating creams; line of hair products (shampoos and conditioners); facial and body creams; facial serum and raw materials and ingredients.

The implementation of the methodology was carried out successfully, no more animal tests are carried out for the evaluation of skin and eye irritation in Bagó. We are currently working on the implementation of an *in vitro* approach for the evaluation of skin sensitization to definitively replace the use of animals in the evaluation of cosmetic products.

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Training and implementation of a reconstructed human epidermis (RHE) model to evaluate skin irritation in Argentina

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The implementation of alternative methods to the use of animals for regulatory purposes in Argentina is incipient. Although there are still no restrictions at the regulatory level, social pressure and advances in regulatory matters in other countries have led to the need for non-animal methodologies for the evaluation of cosmetic products. Faced with the global trend of replacement, the first Alternative Methods Laboratory (LMA-EBAL) was created in Argentina with the purpose of implementing, developing and training *in vitro* methodologies. In this sense, we organized the first hands-on workshop of SkinEthic™ OECD TG 439 for Skin Irritation Test in the country. The training was ministered by Rodrigo De Vecchi and María Laura Gutiérrez; it was attended by regulators and specialists from the National Administration of Medicines, Food and Medical Technology (ANMAT), from the ANLIS Malbrán Institute, CONICET researchers, professors from the UBA, from the pharmaceutical and cosmetic industry, test laboratories and representatives of the Argentine Chamber of the Cosmetic and Perfumery Industry (CAPA).

In the first implementation of the methodology in the LMA-EBAL, the skin irritation potential produced by 14 AIN VEGAN cosmetic products was assessed according to the OECD TG439 guideline. The success of this experience is important for Argentina since the training was very popular and the implementation allowed testing the SkinEthic import system from Episkin Brazil.

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Establishing confidence in NAMs for developmental and reproductive toxicity

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Evolving considerations around validation of New Approach Methodologies (NAMs) have led to an increased emphasis on more flexible, fit-for-purpose approaches that are tailored for regulatory contexts of use. Qualification of an assay involves establishing scientific confidence to a level sufficient for specific decision frameworks, and depends upon essential elements such as robustness, reproducibility, relevance, and independent review. Testing strategies that are targeted towards screening and predicting developmental and reproductive toxicity potential are often designed to address specific mechanisms or key biological processes that are essential to normal conception and fetal development. It is therefore critical to identify reference data that are also accordingly targeted and, ideally, have demonstrated applicability to humans. Work is ongoing to mine scientific literature, databases, and screening programs to postulate reference chemical lists that are well-substantiated and biologically characterized. Understanding predictive value and appropriate application may differ under scenarios of rapid response data generation, lead compound screening, chemical hazard and risk assessment, and various other contexts of use. Applying systems modeling strategies such as *in vitro* to *in vivo* extrapolation to derive exposure-driven margins of safety can support increased confidence in regulatory application. Current efforts and future directions in qualifying NAMs for use in DART testing rely upon coordination across international partners and diverse stakeholders.

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Is "release" becoming another R? An overview of US laboratory adoption laws and legislation

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In the past ten years laws governing post-research placement for dogs (and sometimes cats) have been passed by fifteen US states and several other states have seen legislation introduced. Howev-

er, information on law compliance and the number of animals released for adoption in these states is lacking.

Cruelty Free International conducted a review of state laboratory laws and, as a case study, surveyed California laboratories covered by the state's laboratory adoption law with a series of questions aimed at measuring the laws' impact. We concluded that without specific reporting requirements and publicly available information about research facilities, their adoption policies and availability of adoptable animals, it could be difficult if not impossible, to enforce such laws or to measure their life-saving impact.

Our review informed federal legislation, the Companion Animal Release from Experiments (CARE) Act that would establish a national requirement for research institutions that receive taxpayer funding via the National Institutes of Health to establish transparent adoption policies for dogs, cats, and rabbits who are no longer used for research and to maintain publicly available data on the program. Like most state-level bills, the CARE Act allows research facilities to determine which animals are suitable for adoption, and coordination with local animal rescue groups or shelters is optional.

Cruelty Free International believes it is not enough to have the homing of animals on a state-by-state or case-by-case basis. We believe that passage of the CARE Act has the potential to save hundreds of dogs, cats and rabbits.

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Establishment of a physiologically based pharmacokinetic modeling for benzene exposure assessment in children population

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Benzene, readily detectable in the ambient air worldwide, is emitted from the storage facilities and products of petroleum. Previously, we reported that benzene was one of the highly concerned volatile organic compounds for the residents living near petrochemical industrial parks. Benzene is also detected in the exhaust of motor vehicles and cigarette smoke. Therefore, it is urgent to monitor benzene exposure in the general population. S-phenyl mercapturic acid (SPMA), a urinary metabolite of benzene in human, is considered as a specific biomarker for benzene exposure. In our present study, we adapted a physiologically based pharmacokinetic (PBPK) modeling for benzene and validated the model with human data from literatures. We further optimized the model with physiological parameters of children and incorporated variation of the parameters into the model. Finally, we validated the model with personal benzene exposure data and urinary SPMA among a children population living near petrochemical industrial parks. This validated model will allow us to utilize SPMA as a benzene exposure marker for risk assessment among the children population in the future.

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c-Src phosphorylation is a key molecular event in distinguishing between irritants and non-irritants on human skin equivalent model

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Topically applied chemicals often cause skin irritation in humans. Several studies have used human skin equivalents (HSE) to identify increased expression of genes to common chemical irritants. However, these gene signatures consist of many genes, making them difficult for high-throughput analysis. We hypothesized that analyzing the upstream signaling cascades would enable us to determine specific kinases that trigger irritation pathways. In this study, we applied irritant and non-irritant chemicals to HSE for 15 minutes and performed a phosphokinase array to distinguish irritants from non-irritants. Most kinases displayed no difference in phosphorylation status between treatments with irritant and non-irritant compounds. However, an abundance of phospho-c-SrcY419 was increased upon topical application of a known irritant lactic acid (LA), in comparison to the non-irritants methylparaben (MP) and cocamide diethanolamine (Co-DEA), or water applied as carrier control. Previously, we identified a seven-gene signature panel that distinguishes irritants from non-irritant. Four genes identified in this panel (IL-6, PTGS2, MAP3K8, MMP-3) are regulated by phosphorylation of transcription factors AP-1 (c-Fos/c-Jun) and p65/NFκB. In line with these data, we found increased phosphorylation of both c-JunS63 and p65S536 in response to irritants but not non-irritants. In addition, an anti-irritant chemical com-

pound, betaine trimethylglycine could inhibit this phosphorylation of c-SrcY419 and also indicates that c-Src phosphorylation at the Y419 position specifically triggers irritation response. Therefore, c-Src phosphorylation can be used as a marker for use in predicting pharmacological and cosmetic chemical irritation potential in a more robust, rapid, and high throughput manner.

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Non-invasive methods for early humane endpoint determination in a mouse model for colorectal cancer

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A key aspect of improving welfare of laboratory animals is the determination of early humane endpoint criteria for timely euthanasia before severe suffering occurs. Especially non-invasive and less stressful methods should be considered for humane endpoint determination.

Data-based welfare assessment was performed in an orthotopic mouse model for colorectal cancer. Non-invasive behavioral and physiological parameters such as burrowing behavior, nesting activity, body weight, distress score, perianal temperature and mouse grimace scale were evaluated during cancer progression and their accuracy for welfare assessment was directly compared to a continuous monitoring of activity, body temperature and ECG by telemetry.

All welfare parameters indicated no significant changes compared to healthy mice until day 20 during cancer progression. However, 1-2 days before reaching the individual humane endpoint, most parameters changed significantly and indicated an impairment of animal welfare. The non-invasive parameters distress score, burrowing behavior and mouse grimace scale displayed a high accuracy for humane endpoint prediction similar to the sensitive telemetric parameters activity and body temperature.

Accurate early humane endpoint prediction in a murine colorectal cancer model is feasible with non-invasive and less stressful methods.

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Severity assessment of bile duct ligation models

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Purpose: The goal of this study was to investigate, if partial bile duct ligation (pBDL) still enables scientists to explore pathological features of cholestasis, while causing less distress to mice than common bile duct ligation (cBDL).

Methods: After induction of partial or total cholestasis in mice the wellbeing of these animals was evaluated by assessing burrowing behavior, body weight and a distress score. To compare pathological features of these animal models, the plasma levels of liver enzymes, liver necrosis and fibrosis were assessed on day 14.

Results: Mice after pBDL had a higher survival rate and their wellbeing was significantly better when compared to cBDL animals. However, only cBDL animals had significantly elevated liver enzymes such as aspartate and alanine aminotransferase. Moreover, the surgical intervention for pBDL was more difficult and prone to reducing the blood flow of the adjacent artery. This has a major effect on the observed pathological features such as the extent of necrosis and collagen deposition.

Conclusion: Therefore, pBDL might be the preferred animal model when considering animal welfare but has also specific disadvantages when compared to cBDL.

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A Swedish strategy to replace animal experimentation

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The Swedish National Committee for the Protection of Animals Used for Scientific Purposes and its executive body the Swedish 3Rs Center have set out to compile a strategy to replace animal experimentation in a Swedish context.

The project was kicked-off through an open workshop where stakeholders were invited to give input. Four different topics were discussed: positive attitudes towards development and implementation of replacement, how science communities can collaborate to drive the transition from animal use to alternative methods, areas where animal experimentation could be replaced in a near future, and finally, what obstacles are seen in the validation and evaluation process for new methods.

The discussions focused on funding, collaboration between researchers and the importance of visualizing information on non-animal methods. The information has been processed by the National Committee and the 3Rs Center and compiled into a working document.

Three overall focus areas have been postulated:

- Knowledge and collaboration on animal-free research shall increase
- Animal-free methods shall be developed and evaluated at a faster pace
- Animal-free methods shall be implemented and replace animal experimentation as soon as scientifically possible

The project has now moved into the next phase – anchoring with the target groups. During this process, input will again be collected from stakeholders and the document modified to encompass their views. Ideas for specific interventions or activities will also be collected.

When finalized, the document will be used to direct the Swedish National Committee's work to replace animal experimentation and disseminated to other organizations as inspiration.

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Not quite there: BASF's experience in replacing the six pack toxicity tests for agrochemical formulations

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Several NAMs addressing the so-called six-pack endpoints have gained regulatory acceptance but their predictive capacity for agrochemical formulations is usually not well understood upon adoption.

We report here our experience using *in vitro* methods or non-testing approaches for eye irritation/ serious eye damage, skin irritation/ corrosion, skin sensitization, and acute oral, dermal and respiratory toxicity.

Eye irritation / serious eye damage: None of the evaluated protocols (HetCam, BCOP (OECD 437), two modified BCOP protocols, ICE (OECD 438), EpiOcular™ ET-50) was sufficiently sensitive to predict GHS Cat 1 agrochemical formulations correctly. The EpiOcular™ EIT (OECD 492) was predictive for non-irritants formulations.

Skin irritation / corrosion: Comparing *in vivo* and *in vitro* skin irritation and corrosion data indicates a lack of applicability of the current protocol of the *in vitro* skin irritation test (OECD 439) for agrochemical formulations.

Skin sensitization: Our experience with *in vitro* skin sensitization tests for agrochemical formulations is limited to only a few formulations that were assessed in the "2 out of 3 approach" (OECD 497) comprised of the DPRA (OECD 442C), the LuSens (OECD 442D), and the h-CLAT (OECD 442E).

Acute oral, dermal and respiratory toxicity: Comparing experimentally derived classification to the GHS additivity approach for acute oral, dermal and respiratory toxicity used for formulations containing at least one toxic ingredient falsely characterized the hazards of formulations with interacting ingredients. A proper application of the GHS additivity formula first must ensure the toxic chemical(s) in the formulation do not interact with other ingredients of the formulation.

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Evidence-based severity assessment in epilepsy models as a basis for refinement?

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For ethical and legal reasons evidence-based severity assessment is essential. Within the framework of the research group "Severity Assessment in Animal Based Research" (DFG FOR 2591) suitable parameters for an objective severity assessment are identified and validated. Multidimensional schemes are then used for evidence-based assessment of possible refinement measures. In this study, we focused on chronic epilepsy models in adult mice and rats.

Different behavioral and biochemical parameters were evaluated in mouse and rat kindling and post-status epilepticus models. In some sub-studies, sleep patterns, locomotor activity, heart rate, and heart rate variability were analyzed based on telemetric re-

cordings. Selected parameters were applied to assess the potential benefit of telemetric over tethered recordings as a refinement measure.

Parameters that stood out as sensitive indicators of animal distress in various sub-studies include burrowing, saccharin preference, fecal corticosterone metabolites, heart rate, and heart rate variability. In addition, nestbuilding and grimace scores were affected in the early post-surgical phase in both species. Comparison between telemetric and tethered recording approaches in rats did not reveal major group differences.

In conclusion, we identified various valuable and sensitive severity assessment parameters suitable for application in epilepsy models. Interestingly, cross-correlation analysis argued against an added informative value of telemetric parameters suggesting that a combination of behavioral and biochemical parameters is sufficient to capture sensitive information about an animal's distress and the affective state. Concerning refinement, our findings indicate that telemetric recordings only offer small advantages compared to cabled set ups in rats.

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Animal welfare assessment and early humane endpoint determination in animal models for pancreatic cancer

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Preclinical oncological *in vivo* research remains essential to obtain new treatment strategies. Effective refinement of animal models in this research area requires data-based assessment of the specific cancer burden.

Data-based animal welfare assessment, including parameters such as body weight, distress score, nesting activity, burrowing behavior, perianal temperature, fecal corticosterone metabolites, plasma corticosterone and mouse grimace scale was performed during the induction and progression of different murine pancreatic cancer models.

Significant changes of burrowing behavior, body weight change and perianal temperature indicated a short-term, mild impairment of animal welfare either during orthotopic, intravenous or subcutaneous injection of murine Panc02 cells. Hardly any changes of the welfare parameters were observed until day 38 of cancer progression. However, 24-48 hours before reaching the individual humane endpoint (Day 40-77), most parameters indicated an obvious impairment of animal welfare. Especially burrowing behavior and mouse grimace scale had a high accuracy for early humane endpoint prediction in murine pancreatic cancer models.

Data-based animal welfare assessment was successfully implemented to quantify the burden of pancreatic cancer in different mouse models and to determine early humane endpoint criteria.

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Development and deployment of computational tools in drug discovery

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Developing new drugs takes 10-15 years with an average cost of 1-2 billion dollars and an attrition rate of 90-95%. Over the last decade, there has been increasing interest of the pharmaceutical industry for *in silico* tools that could identify key issues of drug candidates early on in the drug discovery process. The objective of this communication is to present examples of computational tools deployed at UCB across the entire pharmaceutical life cycle, based on the Build, Borrow, Buy concept. Particular emphasis will be given to safety aspects. An internal predictive platform containing about 33 predictive models (covering many endpoints, e.g., hERG blockade, mutagenicity, hepatotoxicity, clearance, …) has been built. The platform enables informed decision making and continuous learning from internally generated data to make more accurate predictions. The Borrow pillar consists of external collaborations or participation to consortia (EIT Digital, IMI,…) to share data in the pre-competitive space, pool knowledge and data to improve predictivity of the *in silico* tools, and spread cost and risks associated to their development. Finally, the Buy pillar refers to the acquisition of commercially available computational tools that allows rapid implementation and are well validated and accepted by regulatory authorities. This computational strategy helps to guide design of molecules and prioritize chemical series by predicting their physicochemical, pharmacokinetic, and safety properties before compounds are even synthesized. It reduces attrition rate and improves effectiveness by identifying better drug candidates faster, and also contributes to reduce animal experimentation.

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Assessing photosensitization of chemicals using an *in vitro* approach

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Adverse reactions to substances contained in household products, personal care and cosmetics formulations, and even pharmaceutical products include photosensitivity reactions (photoirritation and/or photoallergy). These reactions occur when the application of a chemical product, topically or systematically, is simultaneous with sunlight exposure. On the other hand, to ensure the safety of these products, phototoxicity and photoallergy assays should be performed. In this sense, the development of new alternative tests to animal experimentation to predict these adverse reactions is of especial interest for cosmetics due to the European ban on animal use and for the other products also necessary to replace animals. In this work, the conditions of an *in vitro* test have been fine-tuned with the final objective of being able to identify chemical products with photosensitizing capacity and to distinguish to the photoirritant activity. For this, the OECD TG432 guideline has been followed with slight variations. Thus, the commercial line of human keratinocytes HaCaT has been exposed to different concentrations of Chlorpromazine (CPZ) and Sodium dodecyl sulphate (SDS) in the absence and presence of UVA light, and cell viability has been determined by the NRU and MTT assays. The results show that both at a dose of 4 J/cm^2 and 5 J/cm^2, CPZ is classified as a phototoxic product. Once the appropriate irradiation dose has been determined the study has included different products with known phototoxic capacity and the conditions to evaluate the photogenotoxic potential has been settled oud. These assays can be a promising simple method to identify photosensitizers.

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Using FBS-free media in *in vitro* cell cultures: Case studies in transitioning and characterizing A549, EA.hy926 and THP-1 immortalized cell lines

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The use of fetal bovine serum (FBS) in cell culture media may hinder the reproducibility and human-relevance of *in vitro* research and is associated with ethical and legal challenges. Increasingly, scientists are focusing on replacing the use of FBS as a supplement in cell culture media with animal-component-free media.

As case studies, A549*, EA.hy926 and THP-1 cells – which are common models for human epithelial alveolar, endothelial and monocyte cells, respectively – were used to demonstrate the process of transitioning cells cultured in medium containing FBS to commercially-available media without FBS.

To determine whether the transition was successful, cellular morphology and functionality were assessed by imaging (scanning electron microscopy); calculating cell doubling time, cytokine release (Bio-Plex), and cell viability (Alamar blue assay). Our results show that, while success varies based on the transition process and type of media, animal-derived components can be replaced in the culture of A549, EA.hy926 and THP-1 cells. Because FBS-free media can replicate the phenotype of the cells similar to that observed in FBS-supplemented medium or a phenotype more similar to normal human cells, the medium should be chosen based on the objective of the study. These case studies can be used as a guide to transition other cell types to FBS-free media.

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The alternative cardiac *in vitro* system: Role of the biomechanical structure and cell composition in the modulation of toxicity response

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Three-dimensional (3D) culture systems have been developed to restore *in vivo* conditions mimicking multicellular micro-tissues. It has been repeatedly demonstrated that only 3D technologies using co-cultures are able to reproduce key aspects of the phenotypical and cellular heterogeneity as well as microenvironmental aspects of tumor growth.

This work aims to compare a 3D co-culture of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs, 80%) and Human Coronary Artery Endothelial Cells (HCAECs, 20%) versus the gold-standard cell system of 2D hiPSC-CMs (100%), placed in contact with Doxorubicin. The 3D model was established by culturing cells into photo-crosslinked gelatin methacryloyl networks. Gel viscoelastic properties were thoroughly studied by rheology. A dose-response curve (0.001-20 μM) was performed, and cells were treated for 7 days. The cell functions (mitochondrial activity, oxidative stress, apoptosis, cell integrity, cardiac and specific cell markers) were characterized.

Our results highlighted the increased resistance of the 3D system to Doxorubicin toxic effect with respect to the 2D culture and showed how mitochondrial activity and oxidative metabolism seem to be the most sensible networks to chemical toxicity. The integration of biomechanical and cell stimuli could favor a more precise evaluation of the toxicity profile of chemicals, supporting a reliable transferability of information from the bench to the bedside.

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Refinement of pain management: Multimodal analgesia for neurosurgical approaches in mice

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In the past the rare application of multimodal perioperative analgetic regimens raised concerns about the quality of pain management in animal-based neuroscience research, which is often requiring intracranial implants. The aim of our studies was to assess the development during a recent decade based on a systematic review and to develop a multimodal analgesia approach.

The systematic review compared analgesia approaches for mice and rats reported in studies over a decade. Efficacy and tolerability of a multimodal analgesia regimen combining an NSAID (carprofen), sustained release buprenorphine and/or a local anaesthetic (bupivacaine) was determined in C57BL/6J mice with implantation of an intracranial electrode. Postsurgical pain was assessed based on a multidimensional pain assessment approach combining behavioral, grimace scale, and biochemical parameters.

Our systematic literature search revealed that 75% of studies from 2019 failed to report perioperative analgesia use and that monotherapeutic approaches were still much more common than multimodal approaches. Data analysis from the *in vivo* study indicates more pronounced changes of different behavioral parameters in mice receiving NSAID monotherapy. Among other findings these mice show a lower body weight as compared to groups with multimodal analgesia.

In conclusion, our findings demonstrate that lack of analgesia and oligoanalgesia remains an issue in neuroscientific research. Thus, further educational efforts seem to be necessary emphasizing the relevance of optimized analgetic approaches as a refinement measure with consequences for animal welfare and study quality. First findings from the analgesia study indicate superiority of multimodal approaches for murine craniotomies.

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Evaluating fish acute toxicity of nanoparticles by a modified version of the OECD Test Guideline 249

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Microfibrillated cellulose (MFC) and silica nanoparticles (SiO2NP) are nanomaterials with several potential applications in nanotechnological products. The MFC renewable origin and biodegradability properties have attracted attention in sustainable product development; however, its environmental impacts are not yet fully understood. In this study, the fish acute toxicity potential of MFC and SiO2NP was evaluated by a modified version of the OECD Test Guideline 249, which ensured bioavailability, avoiding death caused by deposition on cells and not due to proper cell uptake. Thus,

RTgill-W1 cells were exposed to MFC (39 to 1250 mg/L) and SiO2NP (7.81 to 250 mg/L) in 24 well-plates for 24 h under conventional exposure conditions (static exposure) and orbital shaking; cell viability was quantified according to the OECD 249. Controls (cell-free, solvent, and positive controls) were in accordance with the validity criteria of the test. No effect on cell viability was observed for MFC in both exposure conditions. For SiO2NP, concentration-dependent toxicity was verified, and cell viability was decreased when static exposure (lower values of EC50) was compared to the orbital shaking condition due to the higher deposition of SiO2NP on gill cells and not due to cell uptake. In the orbital shaking, the most conservative EC50 value was in the CFDA-AM test (70.51 mg/L), which classified SiO2NP as slightly toxic to aquatic life. In conclusion, safer SiO2NP concentration needs to be established for nanotechnology applications. Moreover, it was shown that the experimental design to estimate nanoparticle toxicity by *in vitro* methods correctly should consider exposure conditions.

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Skin irritation of nanoparticles using a reconstructed human epidermis

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Purpose: Microfibrillated cellulose (MFC) and silica nanoparticles (SiO2NP) have been proposed to the development of sustainable and renewable products. MFC presents a thickener property and allows interactions with different materials, and SiO2NP can be used as a chemical carrier. Considering the lack of knowledge on nanoparticle toxicity, this study aims to verify whether MFC and SiO2NP are skin irritants after acute and repeated exposure using a reconstructed human epidermis (RHE).

Methods: The skin irritation test was performed using an in-house RHE model. Exposure of MFC (1%) and SiO2NP (0.5%) followed the SkinEthic protocol described in the OECD TG 439, with adaptations for the repeated exposure. Cell viability was measured by the MTT assay, and enzyme-linked immunosorbent assays with RHE supernatants were performed to evaluate proinflammatory cytokines (TNF-α, IL-8 and IL-6). To evaluate nanoparticle skin permeability, an autofluorescence assay was performed using confocal microscopy.

Results: According to the OECD TG 439, an irritant response is defined by a threshold of cell viability lower than 50% compared to the negative control. Our data showed that both nanoparticles are not potential irritants in acute and repeated exposure. No significant difference was found in the cytokine production compared to the negative control. Both tested nanoparticles did not permeate the epidermis barrier and no histological changes were observed. Therefore, this study showed that MFC and SiO2NP are not skin irritants and may be used as sustainable alternatives to developing nanotechnological products.

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An exposure-led approach to worker safety assessment of sodium 2-hydroxyethane sulphonate using new approach methodologies

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Next Generation Risk Assessment (NGRA) is an exposure-led approach to safety assessment that uses New Approach Methodologies (NAMs). The scientific principles of NGRA were applied to assure the safety of workers in factories handling sodium 2-hydroxyethane sulphonate (sodium isethionate, SI).

The worst-case levels of exposure of workers to SI in several factory environments were estimated using factory-specific data and occupational exposure models. These exposure values were then used to estimate levels of systemic exposure to SI following occupational exposure using Physiologically Based Kinetic (PBK) modelling. Experimental ADME data from NAMs were also generated on SI for this PBK modelling which indicated a worst-case plasma Cmax of 0.8 µM across the entire life cycle of SI.

The bioactivity of SI was assessed in a battery of NAMs relevant to systemic, reproductive, and developmental toxicity. Concentration-response curves were derived for 40 cell stress markers and High Throughput Transcriptomics was conducted in HepG2, HepaRG and MCF7 cells. Pharmacological profiling of SI against 73 targets was conducted as well as specific assays relating to developmental toxicity (Reprotracker, devTOXqp). Points of Departure (PoDs) for SI in these assays ranged from 104-5044 µM.

Cmax values obtained from PBK modelling of occupational exposure to SI were compared to PoDs from the bioactivity assays to derived Bioactivity/Exposure Ratios (BER) which demonstrated the safety for workers exposed to SI. This work provides additional evidence to support the application of NGRA for regulatory purposes such as REACH.

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Systematic reviews of animal studies: The positive impact on research

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The Netherlands Organisation for Health Research and Development, ZonMw, has funded training and coaching in performing preclinical systematic reviews since 2012. To evaluate the impact of this funding, we assessed the effect of performing systematic reviews on researchers and their research during the period 2012-2020.

An online survey was sent to grantees to investigate their experience of conducting a systematic review and its impact on their attitudes and subsequent (animal) research. In-depth interviews were conducted with a sub-group.

Respondents reported that conducting a systematic review changed their views on the quality of animal studies generally, with some leaving animal research altogether to focus on alternatives, meta-research or open science. They related how they shared their newly acquired knowledge with their teams and used their new insights to improve their subsequent studies, e.g., through better design, conduct and reporting. They reported advocating for change among their peers, e.g., by publishing opinion papers, and explained that their review findings inspired new primary animal and human studies as well as preclinical systematic reviews. Participants suggested their field would benefit if more researchers were able to conduct and understand the value of systematic reviews.

Training and coaching in systematic reviews positively impacted animal research quality, transparency, and awareness in this case study. Therefore, other funders should follow this example to encourage systematic reviews, as a greater use is likely to stimulate the further spread of good practice in animal research and facilitate the transition to animal-free alternatives.

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Make my skin care trusted! How to reliably scale biological screening in cosmetics using multiomics approach

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Latin-American industry seeks to take advantage of the biodiversity and the huge potential of nature as a distinctive and essential component, considering the cosmetic industry one of the main stakeholders. The research of new cosmetic actives is a process that involves the use of several types of chemical, biochemical, and molecular analyses to identify the true biological potential of these materials for humans, particularly if they are from a natural source.

The application of high-throughput analytical methods such as transcriptomics and proteomics, associated with bioinformatic tools shows a huge potential to generate feasible and human-relevant data in the exploration of these new materials. The integrations of these tools are going to achieve the optimization of time and resources and can be extrapolated to dermatological research. In our work, we associated the results obtained from transcriptomic assays with proteomic assays to identify the current potential use of raw materials with cosmetics applications relevant to humans. We were able to establish the most relevant biological pathways ingredients, as well as identify alternative uses within a dermal application scheme. Moreover, we find thresholds where the transcriptomic results are not relevant because these cannot be seen at the molecular level and are not noticeable for consumers. The development and breakthrough of Latin-American science and industry depends on the implementation of novel methodological approaches that allows us to obtain more human data relevant in short time to give strong scientific support to new findings. That is why important to universalize access to these emerging technologies.

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Fostering awareness, accessibility, and acceptance of animal-free antibodies

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Most antibodies used in research are derived from animals, with about 1 million animals used in their production each year in Europe [1]. The emerging animal-free production techniques, like phage display, can allow improved reproducibility for research, diagnostic, and therapeutic purposes, as well as having potential for greater functionality and upscale [2]. These techniques are gaining worldwide recognition as the way forward in biomedical research.

However, there is a general lack of awareness around this topic. As animal-free antibodies are relatively "new", it raises questions around their validity, affordability, and quality, even when researchers are aware of them. Many suppliers and researchers who are aware are hesitant to change materials or techniques that are well established without good cause.

The European Centre for the Validation of Alternative Methods (ECVAM) published recommendations that "animals should no longer be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic application" [1]. As part of this initiative, Animal Free Research UK will launch an online animal-free antibody database in April 2023. This platform aims to encourage researchers to prioritize the use of animal-free antibodies while facilitating their access. We hope this will be a strong basis for creating more impetus towards acceptance of animal-free biomaterials, and for building more informative and collaborative resources as a support for scientists embracing human-relevant research.

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How the analysis of target organs in cosmetics could prioritize the NAMs development?

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A major point in the safety evaluation of cosmetic ingredients includes repeated dose toxicity testing, which is intended to address the most complex human endpoints. On this tenth anniversary of the full implementation of the animal testing ban for cosmetic ingredients in the EU, no alternative solutions are available to fully replace repeated dose toxicity testing.

In the New Approach Methodologies and Next Generation Risk Assessment race, the main purpose is to determine the fate of chemicals after they potentially reach the systemic circulation and arising specific target organ/systemic toxicity coming from repeated exposure.

In this context, it seems important for cosmetic industry to prioritize the development of alternative methods on target organs and to develop promising tools for the contextualization of Generic Cellular Effects at the organ level.

From the analysis of different *in vivo* studies data sources: literature, toxicity databases, Cosmos and internal toolbox, a meta-analysis has been performed including various study types.

Preliminary analysis demonstrates that most organs were not impacted by cosmetic ingredients. Furthermore, the top three most affected organs were the liver, the hematological/spleen system and kidneys although with markedly lower occurrence. From that exploration, we provided more comprehensive data regarding influence of parameters such as study length, cosmetics categories, or exposure route on *in vivo* behavior of ingredients.

These results could be used to orient and justify the choice of models from both *in vitro* and *in silico* tools to increase systemic toxicity expertise and sustain decision making for safety evaluation of new ingredients.

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Discovery of critical transcription factors in anthracycline-induced cardiotoxicity

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Cancer treatment has drastically evolved throughout the years leading to an increasing number of cancer survivors. Anthracyclines (ACs) continue to play a prominent role in standard care ever since their emergence in the 1960s. However, the clinical use of ACs is restricted due to dose-dependent cardiotoxicity that can ultimately result in heart failure, which is evident in up to 9% of AC-treated patients. Currently, there are no clinical biomarkers, and only one drug approved for treatment of antracycline-induced cardiotoxicity (AIC). Improved understanding of the biological mechanisms driving AIC can promote patient stratification strategies and can help developing new and effective interventions to reduce AIC.

Mechanisms underlying AIC are partly driven by changes in gene expression, governed by transcription factors (TFs). Here, we identified critical TFs controlling AC-induced gene expression changes from RNA-seq data generated from AC-treated cardiac microtissues. Affirmingly, a subset of these TFs is linked to cardiotoxicity including TP53 and ATF3. Additionally, TFs not yet linked to cardiotoxicity (FOXO3, AHR and GATA2 among others) were also identified. We selected 27 TFs for further investigation and their importance in AIC was functionally tested by RNA interference. Knock-down of FOXO3 sensitized cardiomyocytes two-fold to AC treatment and knock-down of GATA2 and AHR significantly dysregulated cardiomyocyte mitochondrial function. We are currently investigating the role of these TFs in controlling mitochondrial fitness, DNA damage, and apoptosis after doxorubicin exposure. These efforts will aid in creating a better understanding of underlying biological mechanisms of AIC.

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Demonstration of suitability of *in vitro* antigenicity assay for potency testing of human vaccines

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In the frame of its 3Rs program, Sanofi Vaccines has been developing and implementing *in vitro* antigenicity assays for potency assessment of its vaccines. This applies both to new products under development as well as established products based on recombinant protein, bacterial subunit and inactivated viral antigens. In all cases it is necessary to demonstrate the suitability of the new assays whether or not an existing method is already applied.

After giving a few definitions, we will share some case studies for which suitability assessment has been successfully carried out for a replacement method of an existing *in vivo* potency assay allowing for its regulatory acceptance and implementation for batch release and stability monitoring. We will then describe a standardized process that has been defined encompassing monoclonal antibody/ligand characterization, reference standard selection, stability indication studies, comparative studies with existing method, demonstration of capacity to measure consistently and to detect subpotent lots, leading to a standard submission package for new or established products.

This standardized suitability demonstration approach intends to facilitate regulatory acceptance of *in vitro* potency testing using antigenicity assays worldwide. It is applicable to any product and/or antigenicity assay and will be in particularly useful for vaccines containing diphtheria (D), tetanus (T) and acellular pertussis (aP) antigens which are still currently tested for potency with assays entailing the use of a large number of laboratory animals.

This work was funded by Sanofi.
Emmanuelle Coppens is a Sanofi employee and may hold shares and/or stock options in the company.

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Characterization of hepatic 3D spheroids using multiphoton fluorescence microscopy and OMICS

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As regulatory legislation mandate for hazard assessment of a large number of chemicals, testing for toxicity and bioconcentration has become more crucial. So, the attempts to develop non-animal and alternative bioassays, with a focus on the use of cells to replace *in vivo* fish toxicity tests, have increased, with focus on fish. The SPHERTOX project is assessing the rainbow trout (RT) hepatic 3D spheroids model's suitability as an alternative chronic toxicity and bioconcentration method with enhanced comparability to *in vivo* test systems in fish. The hepatic 3D RT spheroid system is a novel cell model, requiring comprehensive morphological and physiological characterization. We, therefore, investigated the uptake, disposition and toxicity of chemicals in spheroids by using a high-resolution multiphoton fluorescence microscopy (MFM). MFM was used to visualize cellular and subcellular responses during exposure to sublethal concentration of copper sulphate, 17 β-ethinylestradiol, pyrene, 3,4-dichloroaniline and carbonyl cyanide m-chlorophenyl hydrazone. Namely, biomarkers for morphology, area, diameter, sphericity, actin synthesis, cytoskeleton, lipid membranes integrity, bile canaliculi structures, DNA/ nucleus staining, viability, metabolic activity, oxidative stress were assessed. Our findings suggest that the 3D spheroids maintain their morphological and physiological integrity, as well as viability for longer than five weeks post-isolation, with no signs of hypoxia at the core. Furthermore, transcriptomics and metabolomics are being explored to unravel the spheroids physiological complexity and potential use in chemical toxicity studies. The morphology and physiological responses of RT hepatic spheroids observed suggest that this model has significant potential as a future toxicity screening tool for different chemicals.

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Progress in predicting teratogenic potential 10 years after the EU animal testing ban

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On this tenth anniversary of the full implementation of the animal testing ban for cosmetic ingredients in the EU, there are still no alternative methods available to fully replace Developmental and Reproductive Toxicity (DART) testing. Therefore, research into reproductive toxicity and teratogenicity is a priority, with the overall objective of developing and evaluating New Approach Methodologies (NAMs) in an Integrated tested Strategy (ITS) for the evaluation of this endpoint for new ingredients.

We conducted a review of NAMs representing various Adverse Outcome Pathways involved in teratogenicity and evaluated which of these could be employed in an ITS to fill the gaps currently lacking. The objective is to develop a robust toolbox of endpoint assays which captures the cellular processes and signaling pathways underlying the key stages in teratogenicity.

We present here the performance of three NAMs, alone and in combination, for their ability to correctly identify the teratogenic potential of a panel of chemicals. The NAMs were the *in silico* DART QSAR model; the "devTOX quickPredict" assay and the Zebrafish Embryotoxicity Test.

These NAMs, exhibited a good sensitivity and specificity, especially when outcomes from all three assays were combined or used in a decision tree approach for all chemicals. The latter appears to be an interesting predictive approach for evaluating the teratogenic potential of chemicals. Future investigations include refining the assays outcomes regarding metabolism, new methods to obtain a robust toolbox for evaluating teratogenicity. Case studies using this toolbox will help building confidence in this approach.

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Between the idea and the reality: A critical review of the Canadian Council on Animal Care

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Unlike every other G-7 country, Canada stands alone in having little national protection for animals used in science. The Canadian Council on Animal Care (CCAC) – a non-profit organization dominated by those engaged in animal experimentation and predicated upon voluntary compliance – has been tasked with oversight of its member organizations, which are largely confined to public facilities and institutions.

Using Ontario provincial inspection reports (the only province with legislation governing the use of animals in science), firsthand testimony from former animal care committee (ACC) members, information provided on the CCAC web site and the CCAC's own data on how animals are used in research, this presentation will contrast CCAC claims about its mission and activities with the stark reality for animals in the many laboratories, testing facilities and academic institutions that comprise CCAC membership. As an example, the CCAC states that "the animal care committee must be at the centre of the animal care and use program" and "act as a strong and visible advocate for the ethical and humane treatment of animals under its care." Yet an undercover investigation exposed mistreatment of research animals even though there was an in-house ACC. Freedom of information documents from Ontario's Ministry of Agriculture, Food and Rural Affairs exposed absent and dysfunctional ACCs.

We ask – who is protecting research animals in Canada?

The presentation will close with our recommendations on how Canada can bring about meaningful change on behalf of the millions of animals used in experimentation in this country.

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A human model of neurodegeneration – Human organotypic retinal cultures

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An organotypic culture system has been established and characterised using human retina. Such cultures preserve retinal cytoarchitecture and provide an experimental system that is clinically relevant to study both neurodegeneration and neuroprotection in the retina. In glaucoma there is loss of retinal ganglion cells (RGCs), so the model was developed to assess degeneration of these cells. Human ocular tissue was provided by the East Anglian Eye Bank. Eyes donated for corneal transplant with additional consent for research were utilised. The retina was dissected from the globes within 24 h post mortem after removal of the cornea for transplantation. Circular explants were taken from the paramacular area of the retina and cultured in DMEM/HamF12 with no addition of serum. RGC numbers were assessed using immunohistochemistry and quantitative PCR using RGC markers. Apoptosis was assessed via TUNEL labelling. Individual explants were found to have equivalent numbers of RGCs enabling protocols with multiple experimental conditions to be used. The number of RGCs was found to be consistent across donors of different ages and post mortem duration (up to 30 hours). Experimental conditions modelling pathophysiological stresses associated with glaucoma (oxygen-glucose deprivation, glutamate, ATP) caused RGC degeneration, together with neuroprotective strategies, could be assessed. The model was found to be versatile and reproducible. Whilst the model was developed to assess RGC death in relation to glaucoma, it also has the potential for investigation of other retinal pathologies. The major challenge has become access to the human ocular tissue, a key factor limiting research using this model.

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Science of cosmetics' safety assessment

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Cosmetics Europe (CE) has funded and driven comprehensive research addressing the need for human-relevant and exposure-driven New Approach Methods (NAMs) for Next Generation Risk Assessments (NGRAs) of cosmetics ingredients. From 2016 to 2022, Cosmetics Europe ran a science program via a consortium of 14 member companies, the Long-Range Science Strategy (LRSS).

The ambition of this program was to advance the science of safety assessment by mainly leveraging existing tools for decision-making and collaborating with relevant stakeholders. Endpoints addressed were eye irritation, genotoxicity, skin sensitization, ADME/tk and systemic effects. Case studies played a central role in LRSS as means to demonstrate how new approach methodologies (NAMs) could be used for safety assessment, identify gaps and serve as ways to establish a dialogue with stakeholders.

Eye irritation, genotoxicity and skin sensitization are advanced but warrant support of the regulatory approval process.

For systemic toxicity, a 10-step framework for read-across supported by NAMs was developed and an initial toolbox for low tier ab initio assessment was evaluated.

With LRSS, a major step forward was accomplished in the use of new approach methodologies for the safety assessment of cosmetics, and close dialogues were established with the European Scientific Committee for Consumer Safety and the OECD on these developments.

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An avian 3D spheroid hepatic cell assay for monitoring bioactivity related to naphthenic acid contamination in wetlands near tailings ponds in the Athabasca Oil Sands Region

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The Athabasca Oil Sands Region (AOSR) is a major source of oil for Canada and one of the largest bitumen extraction sites in the world. Bitumen extraction produces large volumes of waste called tailings, which have been shown to be toxic to various organisms due to the presence of naphthenic acids (NAs) and polycyclic aromatic hydrocarbons. There is concern that surrounding tributaries and wetlands are susceptible to leaching from the large containment ponds where tailings are stored (i.e., tailings ponds). The objective of the present study was to determine NA concentrations and *in vitro* bioactivity of extracts derived from passive samplers deployed in AOSR wetlands with varying proximity to

tailings ponds. NA concentrations were determined using liquid chromatography-tandem mass spectrometry. Bioactivity was determined in 3D spheroid-cultured chicken LMH cells by cytotoxicity, EROD and gene expression assays. We detected elevated levels of NAs in wetlands close to tailings ponds compared to reference wetlands. None of the extracts were found to be cytotoxic. Next steps include determining EROD activity to evaluate CYP1A induction and measuring gene expression using a chicken ToxChip PCR array. The ToxChip contains a curated list of toxicologically relevant genes. Bioactivity results will be used to estimate the relative avian toxicity of the extracts. Further, bioactivity data will be compared to NA concentrations to determine if they are capable of identifying NA contamination. Ultimately, we aim to demonstrate the utility of a non-animal, *in vitro* screening approach to enhance environmental monitoring efforts in a priority Canadian ecosystem.

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NURA offers free training to advance new approach methodologies in toxicological assessment

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Regulatory agencies worldwide are recognizing the need to shift from animal-based toxicological assessment to human specific new approach methodologies (NAMs). The NAM Use for Regulatory Application (NURA) program is an educational platform offering free webinars and training series on various topics across regulatory toxicology. NURA aims to increase the use of human-specific *in vitro* and *in silico* approaches by raising awareness of new approaches and building confidence in their applications. The free platform allows scientists and regulators to present and ask questions to facilitate the shift to human-specific toxicological assessment.

NURA content includes the on-going DyNAMic Discussion series that receives an average of 248 live views per session. In addition, NURA curates multiple topic focused training series throughout the year on subjects such as PBPK, NICEATM's Integrated Chemical Environment (ICE), Medical Devices, and more. Since its 2018 inception, NURA has offered over thirty individual training sessions. In 2022, NURA hosted 14 of those sessions with a total of 2,425 live attendees. To increase accessibility, all NURA events are recorded and made publicly available for free at prcm.org/NURA, where these archived videos received nearly 1,000 additional views in 2022 alone.

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Acute oral toxicity assessment of pesticides

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Agencies worldwide have attempted to develop nonanimal approaches to predict acute systemic toxicity, but it remains a challenge yet. In Argentina, the rodent acute oral lethal dose (LD50) is demanded for products registration. However, local regulatory organisms and industries are willing to replace the *in vivo* assay. Our goal is to develop an integrated approach for acute oral toxicity assessment of pesticides. The BALB/3T3 Neutral-Red Uptake Cytotoxicity Assay (OECD 129) was used to estimate the *in vitro* LD50 values of 19 pesticides, which were provided by ATANOR S.C.A and GLEBA S.C.A along with their *in vivo* LD50 doses. *In vitro*/*in vivo* datasets were classified as Extremely Hazardous (EH: < 50mg/Kg), Moderately Hazardous (MH: 50-2000 mg/Kg) or Unlikely Hazardous (UH: > 2000mg/Kg). None of the pesticides was classified as EH for both datasets. With the *in vivo* data 84% was classified as UH while 16% as MH. In the *in vitro* assay 11% was classified as UH while 90% as MH. The concordance was 26%, none of the *in vitro* LD50 values were underestimated and 74% was overestimated. *In vitro* results were integrated with the oral bioavailability of the active ingredients, predicted by SwissADME, which explains *in vitro* overestimations. *In vitro* classification is more conservative than *in vivo* one, most of the pesticides were categorized as UH with the *in vivo* data unlike the *in vitro* data that fell into the MH level. Integration of the *in vitro* assay and *in silico* tools represents a first step for the replacement of the *in vivo* assay.

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Reducing and replacing animal experiments: Europe needs a targets-based action plan

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Despite an oft-cited commitment to replace animal experiments in the European Union (EU), and widespread public unease about the scale and persistence of animal testing, there is no proactive strategy to phase out all types of animal experiments in place. Each de-

cade passes with only slow, incremental change at a rate that could see animal experiments persist in Europe for the best part of another century. The standard response is that we cannot end animal experiments until non-animal methods are in place, yet the acceptance of non-animal methods is impeded by the continued acceptance of animal testing methods. It's a Catch-22 argument that Europe will remain trapped in unless the development, validation and use of non-animal methods is given leadership, funding and urgency in the EU, and until we accept that we need to think beyond like-for-like replacements and towards a fundamental change in approach.

In this presentation, we make the case for a targets-based strategy to phase out all animal tests across the EU. We explain why the existing Directive on the protection of animals used for scientific purposes (2010/63/EU) is not sufficient and provide suggestions for change. We calculate how many animals could be saved if reductions were made in particular areas of testing, and suggest legislative, political and funding commitments that could be made to achieve these reductions. Finally, we provide some concrete actions that the European Commission, member states and other organisations could take – many immediately – to bring animal testing to an end.

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The RAT list: A tool for highlighting areas of animal use ready for replacement

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It may be commonly assumed that animal tests that have subsequently been replaced with non-animal alternatives no longer occur, or at least rarely. The reality is that such animal tests can persist and even increase long after the adoption of suitable alternative methods. In an effort to draw attention to this issue, Cruelty Free International has been closely tracking a number of animal tests over the last seven years to identify the scale and cause of this phenomenon.

We created the Replace Animal Tests (RAT) list to highlight ten regulatory animal tests that are still conducted despite having valid non animal replacements. This list includes the rabbit pyrogen, skin and eye irritation and skin sensitisation tests as well as antibody production and various batch safety tests.

In the latest version of the RAT list, we provide evidence that these tests are still being conducted and elucidate the main reasons for their continued use. Some reasons are well understood, such as the desire for international harmonisation before absolute deletion, while others could be due to unclear regulatory requirements or a lack of monitoring and enforcement.

The RAT list is a useful tool to highlight animal tests that are long overdue for replacement, track trends across sectors and regions, educate the public and lobby the relevant regulatory authorities to complete the replacement process once and for all, for both scientific and ethical reasons.

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Incorporating new approach methodologies into regulatory nonclinical pharmaceutical safety assessment

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New approach methodologies (NAMs) based on human biology enable the assessment of adverse biological effects of pharmaceuticals and other chemicals. Currently, however, it is unclear how NAMs should be used during drug development to improve human safety evaluation.

A series of five workshops with 13 international experts (regulators, preclinical scientists and NAMs developers) were conducted to identify NAMs that could satisfy the specific needs of pharmaceutical safety assessments and to discuss how they could be used in this context. Participants generated four maps of how existing NAMs can be exploited in the safety assessment of the liver, respiratory, cardiovascular and central nervous systems. Each map shows relevant end points measured, tools used (e.g., cells, assays, platforms), and highlights gaps where further development

and validation of NAMs remains necessary. Each map addresses the fundamental scientific requirements for the safety assessment of that organ system, providing users with guidance on the selection of appropriate NAMs.

In addition to generating the maps, participants offered suggestions for encouraging greater NAM adoption within drug development and their inclusion in regulatory guidelines. A specific recommendation was that pharmaceutical companies should be more transparent about how they already use NAMs in-house.

As well as giving guidance for the four organ systems, the maps provide a template that could be used for additional organ safety testing contexts. Moreover, their conversion to an interactive format would enable users to drill down to the detail necessary to answer specific scientific and regulatory questions.

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Staying on track: Chemicals regulation and a roadmap for phasing-out animal testing

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The European Commission and the European Chemicals Agency have committed to the development of a roadmap toward the full replacement of animal testing under the chemical's legislation. Such a roadmap has the potential to achieve a lot, including identification of what is needed to enable the use of non-animal approaches, and focusing efforts on new method development and their regulatory integration.

The European Commission are working to revise the key chemical regulations, a substantial undertaking despite the targeted nature of the intended revisions.

We will present the steps that can be taken now which will reduce the long-term burden of revising chemicals legislation and will ensure that chemicals regulations are always in-step with the course set by the roadmap toward the full replacement of tests on animals.

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Sustainable pyrogen testing: Alternatives to compendial assays using animals

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Compliance to regulatory requirements necessitates testing of parenteral preparations and medical devices for pyrogenic substances that may induce life threatening fever reactions in a patient. Traditional pyrogenicity tests applied globally include the animal-based rabbit pyrogen test (RPT) as well as the bacterial endotoxin test (BET), both tests are critically dependent on natural resources, either a living rabbit for the RPT, or the blood cells of the North American horseshoe crab, *Limulus polyphemus* (LAL), and the endangered Asian horseshoe crabs, *Tachypleus tridentatus* or *T. gigas* (TAL). The worlds' increasing concerns with ethics of using experimental animals, and efforts to protect natural resources, coupled with an ever-increasing testing demand caused by the amount of pharmaceutical product testing, led regulatory agencies and pharmaceutical companies to develop and acknowledge *in-vitro* test systems minimizing such dependencies. In this presentation we review suitable *in vitro* replacement tests for pyrogen testing that are acknowledged by worlds' pharmacopeia for ensuring patient safety.

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Engaging Congress to influence agencies: A laser-focus on appropriations legislation

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The United States Congress has incredible influence over federal agencies through Appropriations legislation, which offers an annual opportunity to better integrate nonanimal methods in regulatory policy and reduce animal use in federally funded research and testing. By enacting Appropriations laws, which provide annual funding for agencies, Congress can allocate funding to support the development and use of nonanimal methods. Congress can also include provisions to advance animal-free policy making via report language; although report language is not a legal mandate, it can have a major impact on agencies' policies and spending priorities.

Successful lobbying strategies to secure Appropriations language that favors nonanimal approaches include building relationships with staff in animal-friendly offices, targeting advocacy toward decision-makers on the correct committee, messaging issues persuasively for target offices, and persistently building on prior efforts. In recent years, relevant funding allocations have included $5 million for the Food and Drug Administration (FDA) to reduce animal use across the agency, and $3 million for implementing the FDA's Predictive Toxicology Roadmap. Numerous examples of report language have made progress toward the adoption of nonanimal methods, including encouragement for the Environmental Protection Agency to continue supporting nonanimal methods for chemical safety testing and support for FDA's qualification of nonanimal methods for use in drug development. Engaging with Congress on Appropriations legislation can effectively lead to improved integration of nonanimal methods in agency policies and practices.

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HaCaT and human fibroblasts spheroids: Strategies to reduce FBS in cell culture

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Cells communicate better when they are at a 3D model, mimicking the *in vivo* situation, interacting with an extracellular matrix in a three-dimensional architecture. Although 2D cell cultures are still the most used ones to assess chemical cytotoxicity they do not replicate the cell-cell connections and the cell-extracellular matrix interactions so the spheroid model has been used to replace unnecessary animal experimentations, as they could be formed by single-cells or multicellular. In addition to use the spheroids another current research nowadays is the use of cell culture using animal-free culture media. In this context we proposed the construction of a HaCaT and human fibroblasts spheroid formed by spontaneously process at a non-adherent U-bottom shaped plates, using progressively smaller concentrations of fetal bovine serum (10, 7.5 and 5%), evaluating the quality of the formed spheroids by viability parameters and whether FBS deprivation could delay its formation. The results so far shows that the auto assembling spheroids process is possible and the reduction of FBS could retard the process. Different approaches have been made to improve the spheroids formation using smaller FBS concentration. The adoption of animal-free cell culture conditions could lead to a more reproducible *in vitro* research.

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The use of liver and embryo zebrafish cell lines (ZFL and ZEM2S) as alternative methods to fish acute toxicity and fish embryo toxicity tests

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The adoption of a new method to assess acute toxicity in fish using RTgill-W1 cell line (OECD TG 249) raises questions about using other fish cell lines to increase the alternative methods available for ecotoxicity testing. We investigated the use of two zebrafish (*Danio rerio*) cell lines (adult liver: ZFL; blastula phase: ZEM2S) as alternative methods to the acute fish toxicity (AFT) and the fish embryo toxicity (FET) tests. ZFL and ZEM2S cells in 96-well plates were exposed to twelve different proficiency test chemicals with different modes of action, and four cytotoxicity assays were performed in triplicates (MTT, Alamarblue, CFDA-AM, and Neutral red). For *in vitro-in vivo* comparison, the logIC50 of the test chemicals obtained by each cytotoxicity assay were compared, by linear regression, with the logLC50 values of AFT (96 h) and FET tests (48 and 96 h) obtained in the literature. There was no statistical difference between the results obtained by the different cytotoxicity assays. The ZFL cell line had a higher correlation to fish *in vivo*/embryo test (R^2 = 0.90-0.91) than the ZEM2S (R^2 = 0.64-0.81). Using the regression equations, we transformed the IC50 values into LC50 values and compared the accuracy of classification in US EPA hazard classification for aquatic life. The ZFL had 75% and 88.88% accuracy in the classification considering AFT and FET data, respectively. While ZEM2S had low accu-

racy (41.66-66.66%) considering AFT and FET data. We conclude that ZFL is a promising cell line for use as an alternative method to fish in ecotoxicity studies.

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Analysis of research animal numbers at U.S. Government-funded laboratories

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In contrast to many other western nations, the United States government does not accurately collect numbers of all vertebrate animals used in research or publicly disseminate the limited statistics it currently gathers. While the U.S. Department of Agriculture (USDA) makes annual reports from individual research facilities available via its website, these figures represent only Animal Welfare Act-covered species, excluding most rats and mice, many birds, and all cold-blooded animals. National Institutes of Health (NIH) animal welfare policies apply to all vertebrates, but unlike USDA's annual reporting requirement for the total number of animals bred or housed as well as those used in experiments, NIH requires an "approximate average daily inventory" of animals every four years. Using the federal Freedom of Information Act, we spent more than 12 months collecting 822 forms reported to NIH's Office of Laboratory Animal Welfare that represent animal use at 1,057 individual U.S. laboratories. Our analysis of the records reveals that more than 18.5 million vertebrates are held in U.S. facilities at any given time. However, many laboratories fail to accurately complete the existing form, resulting in the reporting of cages, tanks, or racks instead of animal numbers. We will present on the inconsistencies we found, and the significant effort needed to obtain these data. We will also recommend policy changes to increase transparency around the use of animals in government-funded laboratories, including current efforts we are undertaking with members of the U.S. Congress.

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Evaluating the skin sensitization potential of two endocrine-disrupting chemicals using *in silico-in vitro* test approach

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Endocrine-disrupting chemicals (EDCs) are primarily investigated regarding reproductive and developmental toxicity effects; however, EDCs can significantly alter the immune response. In this study we investigated the skin sensitization potential of two EDCs currently marketed: 4-octylphenol (OP, CAS 1806-26-4), an alkylphenol used for producing phenolic resins, lacquers, cleaning products and pesticides; and a mixture of consisting of diisopentyl and dipentylphthalates (PeP, CAS 84777-06-0), used as a plasticizer. *In silico* tools were used together with several *in vitro* tests including HaCaT cells (quantification of IL-6; IL-8; IL-1α and IL-18 by ELISA), RHE model (quantification of IL-6; IL-8; IL-1α and IL-18 by ELISA) and the THP-1 activation assay (CD86/CD54 expression and IL-8 release). Whereas *in silico* tools (OECD QSAR Toolbox, Toxtree, VEGA) predicted OP as a sensitizer, PeP was predicted as a non-sensitizer. OP clearly activated keratinocytes (increased IL-6 in HaCaT cells and IL-18 and IL-8 in RHE model), while PeP demonstrated potential to activate keratinocytes only in the 2D cell system (increased IL-6 and IL-8 in HaCaT cells). Both tested EDCs activated dendritic cells (OP and PeP increased CD54 and IL-8 expression in THP-1). Overall, the present study showed effective use of an *in silico-in vitro* test approach to investigate the role of OP in the key events (KE) of the skin sensitization Adverse Outcome Pathway (AOP). However, PeP showed conflicting results, as important skin sensitization markers were absent both *in vitro* and *in silico* analyses.

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Evaluating the potential of 4-octylphenol in damaging DNA and causing epigenetic changes by new approach methodologies

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Investigation of the potential of chemical substances to cause DNA damage and alter epigenetic markers is fundamental since these events play a key role in cancer development and progression as well as other human diseases. 4-Octylphenol (OP) is an alkylphenol with surfactant properties presented in cleaning products and pesticides and widely recognized as an environmental contaminant, being currently detected in soil and water samples. OP is already recognized as an endocrine-disrupting chemical (EDC) with estrogenic activity; however, little is known about its potential to cause transient and permanent changes in DNA. Thus, in this study, the potential of OP to cause genotoxicity and alter the global DNA methylation pattern was evaluated by *in silico* tools (QSAR Toolbox, VEGA, Toxtree) and *in vitro* tests with the HaCaT cell line (immortalized human keratinocyte cell line). *In silico* tools predicted OP as non-genotoxic. The *in vitro* results showed that OP (0.5-50 µg/mL – non-cytotoxic concentrations) significantly induced γ-H2AX (a double-strand breaks biomarker) and increased the levels of 5-methylcytosine; both biomarkers quantified by flow cytometry. Additionally, OP significantly increased the generation of intracellular oxygen species (ROS, H2DCFDA by flow cytometry). All the described effects were verified at the lowest tested concentration. Overall, our results pose concern regarding the potential of OP in altering the genetic material of human cells. Therefore, further investigation is needed to better understand the mechanism of action of this chemical substance on the DNA.

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Proteomics in nanomaterial exposure-related cellular toxicity pathway identification

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Background / Rationale / Objective: Enhanced production of engineered nanomaterials (ENMs), namely, silica nanoparticles (SiNPs) with attractive physicochemical properties and consequent applications (e.g. construction materials, cosmetics, food, biomedicine) lead to the likelihood of environmental/human exposures causing health concerns. Health risk assessment of ENM exposures require toxicity information. Currently, toxicity mechanisms underlying the ENM amorphous SiNP exposures are poorly understood, especially, for nanoforms. Meanwhile, application of proteomics in health and disease is gaining interest. Yet, proteomic analysis in chemical toxicity testing for health risk assessment is limited. The objective of this work was to apply high-content proteomics to identify SiNP nanoform(s) exposure-related toxicity mechanisms to support health risk analysis.

Experimental approach: A549 human lung epithelial cells and J774 mouse monocyte/macrophage cells were exposed to well-characterized amorphous SiNPs (dose: 0-100 µg/cm^2) of varying sizes (15, 30, 75 and 100 nm) and surface properties (-C3-COOH, -C11-COOH, -NH2 and -PEG) and secreted/cellular protein responses were analysed by affinity-based protein array and LC-Orbitrap mass spectrometry analysis. In addition, cellular cytotoxicity (LDH-cell membrane integrity; CTB reduction-cell viability; ATP production-energy metabolism) and oxidative stress (GSH/GSSG) status were assessed.

Summary of findings: SiNP nanoform-specific expression changes in cytoplasmic/membrane/mitochondrial and secreted proteins provided information on SiNP-cell interactions, perturbations in cellular metabolism and inflammatory pathways consistent with findings on cellular cytotoxicity and oxidative stress. Also, size, surface groups and agglomeration appeared to be key determinants of cellular cytotoxicity.

Conclusion: These findings demonstrate the value of proteomics in identifying ENM exposure-related toxicity mechanisms that can enable adverse outcome pathway construction to support risk analysis.

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Optimizing physical micro-environment to maintain hepatic stellate cells in quiescence for *in vitro* liver disease modeling

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Hepatic stellate cells (HSCs) are key contributors to liver fibrosis through excessive production of extracellular matrix (ECM) during chronic inflammation. However, studying HSC function has been challenging due to the difficulty of obtaining quiescent HSCs *in vitro*, as they spontaneously activate when cultured on plastic plates. Recent advances in stem cell technology have allowed for the generation of quiescent-like HSCs from human induced pluripotent stem cells (hiPSCs), whose expansion capability is unlimited. However, these differentiated quiescent-like HSCs (iqHSCs) also become activated when cultured using conventional method.

In this study, we developed a culture method to maintain such iqHSCs in a lowly activated state for up to 5 days by optimizing their physical microenvironment. The physical environment of iqHSCs *in vitro* was optimized by manipulating three distinct parameters: mechanical stiffness, ECM composition, and culture dimensionality (2D/3D cultures). For the ECM composition, collagen I and Matrigel were utilized, with the latter being known to potentially reverse activated HSCs to less activated state.

We demonstrated that embedding iqHSCs in a soft hydrogel made of collagen I or Matrigel significantly inhibited their spontaneous activation *in vitro*. Additionally, iqHSCs in an embedment culture with collagen I hydrogel showed the ability to convert to an activated state in response to stimulation of TGFβ1. Hence, our culture method can be used to generate HSCs with functions comparable to that in a healthy liver, paving the way to develop accurate *in vitro* liver models that can fully recapitulate HSC activation for pathogenesis studying and drug development.

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Knowledge from human relevant cell, tissue and mathematics-based methods as key tools for understanding Covid-19

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The European Health Union also addresses the importance to harvest lessons from the coronavirus pandemic improving at EU-level protection, prevention, preparedness and response against human health hazards. It also aims at strengthening EU presence globally, through the EU Global Health Strategy, which guides EU actions for better preparedness and response in the world. Although knowledge from previous SARS infections has been instrumental, not all specific SARS-CoV-2 features have been clarified nor are the underlying molecular and cellular mechanisms fully understood.

It is therefore necessary to understand in detail the dynamics and kinetics of the SARS-CoV-2 virus and its subsequent viral dynamics. The cellular immune response and specific cellular responses to the pathogen can contribute to multi-organ dysfunction. The mechanistic understanding on modulators of the immune response and cell homeostasis balance and specific risk factors is critical to understand the systems biological processes. Cross-community research on SARS-CoV-2 is essential to understand its detailed pathophysiology. Greater investment and innovative methodological approaches are needed to accelerate and continue knowledge gathering on SARS-CoV-2 in all the aspects of the disease. The ambition of the health crisis response work at the European Commission Joint Research Centre is to build according to the CIAO scientific crowd sourcing example (https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/ciao-modelling-pathogenesis-covid-19-using-ad-

verse-outcome-pathway-framework_en) a Science and Technology platform linking scientific domain knowledge, epidemiology, toxicology, bioinformatics, molecular modelling and biomedical ontologies, to support prevention, preparedness and response to (one)health burdens and threats through data science and digital transformation.

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Fractured oversight of U.S. labs: Where's the accountability?

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In the U.S., the Animal Welfare Act (AWA) is the only federal law with legally enforceable standards that governs the treatment of animals in laboratories. However, this law excludes most animals used in laboratories – notably, mice of the genus Mus, rats of the genus Rattus, amphibians, reptiles, fishes, birds bred for experimentation, and agricultural animals used in agricultural experiments. A different U.S. law – the Health and Research Extension Act (HREA) of 1985 – mandates that institutions that receive federal funds must comply with federal policies, principles, and guidelines in their treatment of all vertebrate species used in experimentation, testing, and training – including AWA-exempted species.

While the U.S. Department of Agriculture conducts inspections of laboratories to try to ensure compliance with the AWA, compliance with the HREA of 1985 is based on a system of trust and self-reporting. In particular, institutions that deviate from federal animal welfare guidelines must self-report such incidents to the National Institutes of Health's (NIH) Office of Laboratory Animal Welfare (OLAW).

Using noncompliance reports submitted to OLAW by the top 25 institutional recipients of NIH research funds, this study analyzes self-reported animal welfare violations between 2018 and 2022. We consider the most common violations, and the remedial or corrective actions put in place to mitigate reoccurrence. Despite institutional and federal oversight, the data show chronic and repeated violations of animal welfare guidelines at leading research institutions, with little enforcement or consequential action taken by oversight bodies to ensure compliance with these laws.

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Developing a laboratory mouse animal center in the metaverse – The preliminary practice of visually immersive alternatives to animal testing

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The development of Alternatives to Animal Testing (AAT) technology based on Replacement, Reduction, and Refinement is the direction of the global scientific community's efforts in recent years. Our project uses the existing metaverse technology to solve critical problems in AAT, such as laboratory animal facility guidance, basic maintenance training, and mouse-precise experimental operations. It can create opportunities for interdisciplinary integration of laboratory animal medicine and metaverse technology. Our strategies are as follows: Constructing a VR experimental mouse room, Existing facilities using augmented reality (AR) technology to optimize operation training, Immersive VR technology applied to precise mouse experiment operations, and Promoting AAT with VR and AR technology. Our project combines the expertise in establishing an experimental animal center, mouse operation of the monoclonal antibody production process, and cross-field AAT technology development to explore the possibility of applying the existing metaverse virtual reality (VR) technology to promote AAT in Taiwan. We believe the prototype of the immersive experience for laboratory animal operation can be a forward-looking and exemplary next-generation animal operation training without trauma.

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Extracellular matrix components improve the *ex vivo* expansion of human epithelial melanocytes

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The extracellular matrix (ECM) modulates migration, proliferation, differentiation, and survival by serving as a molecular reservoir and structural scaffold. Different proteins like collagen and Elastin play mostly structural roles, while glycoproteins like Fibronectin (FN) and Laminin provide connections that stabilize the ECM and mediate cell-ECM and ECM-ECM. The ECM in different tissues plays various roles; in stem cell niche maintains tissue regeneration, whereas tumor-derived ECM plays a critical role in oncogenesis and establishes metastasis, promotes cancer cell proliferation, migration, invasion, angiogenesis, and immune evasion. As some cancers like melanoma are too resistant to all regular cancer treatments, it would be interesting to study the ECM components' effect on normal cells like melanocytes to design better treatments in such cases. FN is an ECM component assembled in tissue development and wound healing and contains multiple domains to bind several ECM proteins, growth factors, and small molecules. FN is also considered in the cancer context as it supports proliferation by different mechanisms that reduce sensitivity to apoptosis signals and promote cell cycle progression. Currently, We applied FN as a substrate to investigate its impacts on human epithelial melanocytes, their melanogenesis, and their functional characteristics. Our results indicated the increased expression of MITF and Tyrosinase in the presence of FN, leading to the ascendant amount of melanin production. Moreover, improvement in migration and adhesion ability caused by the interaction with FN indicates new horizons in studying the effect of ECM on cancer treatment strategies, which may be the blocking of the ECM-cell interaction.

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Colon epithelium barrier with vascularized crypts to model inflammatory bowel disease

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Inflammatory bowel disease (IBD) is a complex, debilitating illness primarily affecting the colon. To better study the disease and develop treatments, engineered *in vitro* models with colon-specific physiological features are needed to complement animal models. Current colon models lack integration of colonic crypt structures with underlying perfusable vasculature. Microvasculature is important to incorporate in colon tissues as crosstalk between colon and blood vessel cells is implicated in disease progression. We present a colon epithelium barrier model with patterned vascularized crypts that recapitulate relevant cytokine gradients in both healthy and diseased conditions. Using our previously published IFlowPlate384 platform, we first imprinted crypt topography on fibrin gels using a highly scalable 3D printed stamp with 100% accuracy. Crypts are then populated with colon cells, and we observe proliferative cells spontaneously localized to the crypt niche, just like in native tissue. Colon cells differentiated in our model into epithelial barriers with a tight brush border. Toxicity of the chemotherapeutic drug capecitabine, was tested and showed a dose-dependent response and recovery from crypt-patterned colon epithelium. Perfusable self-assembled microvasculature were then incorporated around the colon crypts followed by treatment with pro-inflammatory TNFα and IFNγ cytokines to model IBD conditions. We found *in vivo*-like stromal basal-to-apical cytokine gradients in tissues with vascularized crypts while IBD vascularized colon tissues provided cytokine cues to instruct immune cell recruitment. Taken together, we demonstrated crypt topography integrated with underlying perfusable microvasculature has significant value for emulating colon physiology and in advanced disease modelling.

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A teratoma *in vitro* model as an animal-free tool to study pluripotency and malignancy of stem cells

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In 2006 it was found that human induced pluripotent stem cells (hiPSCs) can be generated from somatic cells. Like human embryonic stem cells, hiPSCs can be maintained in culture indefinitely (self-renewal) and differentiate into virtually all cell types of the human body. Since they capture the genotype of the donor, hiPSCs are already used successfully for studying mechanisms of disease and for drug testing. However, the presence of residual, potentially malignant cells among the cell population represents a serious safety issue and requires rigorous testing and identification of unsuitable hiPSC lines beforehand.

Importantly, long-term culture of hPSCs leads to their (epi)genetic drift, potentially activating processes that resemble malignant transformation as an adaptive mechanism to the culture conditions. Malignant potential of hPSCs is not fully understood and its evaluation currently relies solely on the assessment of the cells' behavior *in vivo* upon their engraftment into mouse models (Teratoma assay) where hPSCs either form benign teratomas or malignant teratocarcinomas. However, the Teratoma assay is animal-dependent, costly, time-consuming, and only qualitative and has never been standardized.

Since the number of hPSC lines is steadily rising and (pre)clinical studies need tumorigenicity testing, there is an urgent need for a new quantitative animal-free test.

Our work shows data on development and validation an *in vitro* assay based on differentiation and microRNAs which can predict the tumorigenicity of hiPSCs and can therefore replace the *in vivo* assay.

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Safety and efficacy of new UV-filter candidates based on marine compounds

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It's already known that some UV filters can damage coral reefs and marine life and some marine organisms, and their metabolites can be a source of new UV filters and antioxidants. However, their safety and efficacy need to be proved; thus, we evaluated the safety and efficacy of compounds and fraction from antarctic fungus *Aspergillus sydowii* through new approach methodologies to animal experimentation.

The crude extract was subjected to fractionation using vacuum liquid chromatography (VLC) and compounds were isolated from these fractions using HPLC-DAD method. Photostability was evaluated under 9 J/cm^2 UVA radiation. For the safety parameter, the phototoxic (OECD TG 432) and irritant (HET-CAM) potentials were evaluated. For the efficacy parameter, the antioxidant activity was obtained by UVA-induced ROS production in HaCaT cells and in house reconstructed human skin (RHS) model.

One of the studied fractions and its isolated compound presented a broad-spectrum ultraviolet radiation and were considered photostable. Both samples did not present any phototoxic (MPE: 0.0475 and 0.093) nor irritant potential (HET-CAM score 0.25 and 0.50). The fraction used at 1.0 µg/mL showed a statistically significant reduction in the generation of UVA-induced ROS production when compared to the control (approximately 30% reduction). Thus, it is possible to conclude that the studied marine fraction and the isolated compound can be a possible candidates for use in sunscreens since they present a broad-spectrum UV protection and protected from UVA-induced ROS production.

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Photomutagenic potential of UV filters and insect repellents by using photo-RSMN assay

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Insect repellents are considered safe, but there is an increase of their simultaneous use combined with UV filters. In the literature there are some studies that demonstrate interactions between UV filters and insect repellents. Thus, this study evaluated the photomutagenic potential of UV filters and the insect repellent DEET combination in an in-house human skin model, based on Reconstructed Skin Micronucleus (RSMN) assay. The combination of photounstable UV filters avobenzone, ethylhexyl methoxycinnamate and octocrylene with DEET (UVF+D) were diluted in sesame oil and applied onto in house reconstructed human skin models, as well as the positive control 8-methoxypsoralaen (8MOP). After 24 hours the skin models were irradiated and incubated with cytochalasin B until the isolation of the cells, that were dropped onto glass slides, stained and analyzed by fluorescence microscopy. It was observed that the treatment with positive control 8MOP showed statistically higher micronuclei values than the values of the vehicle sesame oil, considered non-photomutagenic. UVF+D presented intermediate values and was statistically equivalent to positive and negative controls. The percentage of binucleated cells observed on skin models treatments submitted or not to UV radiation did not show any significant variation, as well as the cytokinesis-block proliferation index (CBPI). DEET has no evidence of genotoxicity or photogenotoxicity according to TOXNET and HSDB platforms. However, there are some studies indicating that DEET can present mutagenic and genotoxic potential as well as DNA photodamage. In conclusion, the combination of DEET and UV filters presented signs of photomutagenicity that should be further investigated.

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How does stress modify chemical toxicity? A high-throughput *in vitro* screening approach

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Stress plus chemical exposure may produce effects not observed with chemical exposure alone. Therefore, there is a need for new approach methods that can rapidly screen chemicals for their interaction with stressed biological systems, which will advance understanding of inter- and intra- individual susceptibility risk determination. We are developing a method for this using *in vitro* chemical screening by phenotypic profiling of human osteosarcoma cells that express fluorescent nuclear and cytoskeletal fusion proteins (U-2 OS_FP). These cells have glucocorticoid receptors, which is the target of the stress hormone, cortisol. To be able to establish cortisol concentrations during exposure, cells were first transitioned to a serum-free media. During this process, there was a decreased cell proliferation rate as serum, and thus cortisol, levels were decreased. Next, the U-2 OS_FP cells will be co-exposed to a mixture of cortisol and toxicants in a time-course dose-response screen to rapidly evaluate phenotypic changes in cell structure. We are screening 147 chemicals, including chemicals that are relevant to environmental justice (n = 73), impact the glucocorticoid receptor (n = 22) or overlapping gene pathways (n = 25), are carcinogenic (n = 20) or endocrine disruptors (n = 15), and/or induce cell stress responses (n = 13). These chemicals will each be evaluated for whether they have synergistic, additive, or antagonistic effects under conditions of low vs high stress (cortisol) levels. This research will improve human *in vitro* modeling of how stress comorbidity may impact adverse outcomes from chemical exposure. The views are the authors' and do not necessarily represent the views of the U.S.EPA.

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Acute toxicity and wound healing effect of *Xanthium strumarium* L. fruit using animal alternative testing

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Xanthium strumarium L. (XS) fruit, as known as a cocklebur, has been used as a traditional medicine to treat nasal sinusitis, headache, and arthritis due to its bioactive compounds such as glycosides, phytosterols, and phenolic acids in China, America and Europe. Despite its potential clinical uses, most of the research has focused on its anti-cancer and anti-arthritis effects. In previous study, potential of XS fruit as cosmeceutical agent was confirmed increasing hyaluronic acid production and reducing pro-inflammatory cytokines. The aim of this study is to investigate toxicity and wound recovery effect of XS fruit for further potential as cosmeceutical and pharmaceutical agents using animal alternatives models. To confirm its toxicity and wound recovery effect, *C. elegans*, reconstructed human epidermis, and various cell culture models were used for this study.

Acute toxicity was confirmed by 3T3-L1 test and UVB-irradiated *C. elegans*. Although both XS fruits exerted skin wound healing effects, XS-K showed more effective wound closure rate than XS-C in skin reconstructed human epidermis model (RHE) as well as human dermal cells and co-cultured model. In conclusion, both XS fruits from Korea and China had rapid wound recovery in human dermal cells and RHE models and lower toxicity. Thus, XS fruit may be potentially applied in the area of cosmetic and pharmaceutical industries due to its wound healing and lower toxicity.

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An alternative to animal models of epilepsy: Combining human data with computational modelling

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Epilepsy is a debilitating disease characterised by seizures (abnormal electrical activity). It is highly patient-specific where there is significant individualised variation between and within patients. The overwhelming majority of neuroscience research in epilepsy uses animal experiments. Animal models (typically mice & rats) are used to test hypotheses by artificially inducing epilepsy via drugs, invasive electrical stimulation, and genetic modification to produce seizures. Although there is a plethora of data generated, there is very little understanding of the underlying physiological mechanisms. It is controversial whether seizure-like behaviour generated from animal models is even comparable to that found in a human brain *in vivo*.

Purpose: We propose a combination of voluntary human data and computational modelling as a viable alternative to animal models of epilepsy. Specifically, we use photosensitive epilepsy in humans where a seizure-like response is triggered by a non-invasive perturbation (strobing light source) and captured using electroencephalography (EEG). This ubiquitous procedure is quite safe and routinely performed for epilepsy diagnosis.

By mathematically analysing the brain's electrical response to perturbation, we can gain clinically relevant insights into the nature of seizure transitions in humans, without invasive techniques such as electro-stimulation used in animal models. Within this framework we are able to examine changes in brain state before a seizure transition, as well as quantify the individual variability within and between epileptic patients.

This novel multidisciplinary approach moves away from traditional neuroscience research paradigms and aims to minimise and ultimately replace animal models with computational modelling of non-invasive human data.

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Development of a defined approach for eye hazard identification of surfactants according to the three UN GHS categories

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The purpose of this study was to develop a defined approach (DA) for eye hazard identification according to the three UN GHS categories (Cat. 1: serious eye damage; Cat. 2: eye irritation and No Cat.: the absence thereof) for surfactants (DASF). The DASF is based on a combination of Reconstructed human Cornea-like Epithelium test methods (OECD TG 492; EpiOcular™ EIT and SkinEthic™ HCE EIT) and the modified Short Time Exposure (STE) test method (5-min exposure of 0.5% concentration of the test substance).

In a first tier of the DA, an RhCE test method is used to distinguish No Cat. from classified substances. In case of a positive call based on a RhCE method, the modified STE method is used to distinguish Cat. 1 (viability < 20%) from Cat. 2 (viability ≥ 20%). The performance of the DASF was assessed by comparing the prediction results with the UN GHS classification (based on historical *in vivo* data) and with the criteria established by the OECD expert group on eye/skin.

In summary, 91.3% Cat. 1 (N = 23), 66.7% Cat. 2 (N = 9) and 76.0% of No Cat. (N = 17) surfactants were correctly identified resulting in a balanced accuracy of 78.0%. The percentage of correct predictions met the minimum performance values of 75% Cat. 1, 50% Cat. 2, and 70% No Cat. established by the OECD experts. This DA, applicable to surfactants, demonstrated successful discrimination between the 3 UN GHS categories for the identification of eye hazards.

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In silico-in vitro dermal toxicity evaluation of the flame-retardant aluminum diethylphosphinate

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Organic flame retardants (FR) are chemicals applied to manufacture different products to prevent or reduce the flammability of polymers and fabrics. Recent epidemiological studies have shown that dermal exposure is a relevant route of human exposure; however, little is known about the effects of FR on human skin. This study evaluated the dermal toxicity of a recently marketed FR, the aluminum diethylphosphinate (ALPI, CAS 225789-38-8), by an integrated *in silico* and *in vitro* test approach. Thus, skin irritation effects, changes in inflammatory cytokines expression profile (BD CBA Human Inflammatory Cytokine Kit in HaCaT cell line), and generation of intracellular reactive oxygen species (H2DCFDA in HaCaT cells) were the evaluated parameters. HaCaT cells exposed to non-cytotoxic concentrations of ALPI (0.12, 0.06, 0.03 mg/mL) did not induce intracellular ROS nor increased expression of the cytokines. The OECD QSAR Toolbox (v4.5) used mechanistic profilers associated to skin sensitization (protein binding potency Cys and Lys, alerts for skin sensitization; and keratinocyte gene expression). Neither profilers have shown the presence of alerts. These negative results agree with the lack of effect of cytokines IL-6 and IL-8, markers for skin sensitizers in the HaCaT model. However, ALPI has shown a skin irritant potential according to the OECD test method for skin irritation with RHE model, but ECHA reports no irritancy for *in vivo* assays. Actually is known that RHE models are more reliable to determine human skin irritation, but additional studies should confirm its lack of skin sensitization potential and other inflammatory effects on human skin.

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Uncertainty measurement of cytotoxicity index using OECD Document 129

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Despite the use of acceptance criteria in validated methods for safety tests, the evaluation of statistical uncertainties of the results and their impact on the reproducibility and reliability of these analytical tests is limited. Technical and operational variations in intra- and inter-laboratory settings can interfere with the results, and uncertainty measurement calculation is necessary to ensure accuracy and reliability. Measurement uncertainty is a parameter that characterizes the dispersion of the values that can be attributed to the measurement. We applied the Monte Carlo statistical model to calculate the measurement uncertainty for sodium dodecyl sulfate's (SDS) mean inhibitory concentration (IC50) using the OECD Guidance Document 129. An equation relating input and output quantities allowed for the development of the measurement model, using input quantities obtained from the certificates of the instruments used in the test. The Monte Carlo method allowed the identification of the contributions to the measurement uncertainty of the IC50 and their confidence intervals for the *in vitro* cytotoxicity assay recommended in OECD GD 129. The results obtained were in strong agreement. A mean IC50 of 33.78 ± 1.5 µg/mL was obtained, with a standard uncertainty of 1.034 µg/mL and an expanded mean uncertainty of 2.027 µg/mL for SDS, based on a historical series. Metrological evaluations can benefit the reproducibility of new approach methodologies (NAMs) by providing scientists with better assessments of the quality and validity of their results. By quantifying the level of uncertainty associated with measurement, they can make informed decisions based on the data they collect.

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Evaluation of peptide-mycophenolic acid conjugates as new candidates for UV-filters

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Oxybenzone and other UV filters available on the market have some drawbacks to be considered, such as coral reefs bleaching and impairment to different types of marine life. Thus, there is a field for new natural origin UV filters and antioxidants to be discovered, as well as new derivatives combining marine compounds and antioxidant peptides. The aim of this study was to determine the photoprotective and antioxidant potential of antioxidant heptapeptide conjugated with mycophenolic acid. The conjugates were synthesized by semi-synthesis reactions associating the mycophenolic acid with peptides containing the amino acids tyrosine and tryptophan. UV absorption, photostability, phototoxicity (OECD TG 432), and irritation (HET-CAM assay) potentials were evaluated as well as antioxidant potential through the UVA-induced ROS (Reactive Oxygen Species) production in monolayer and RHS (Reconstructed Human Skin). The UV absorption analysis showed that both conjugates presented good absorption in the UVB region and synergistic effect of mycophenolic acid and peptide conjugates. The substances were considered photostable when submitted to irradiation of 27.6 J/cm^2 and did not present phototoxic potential (MPE < 0.100). The conjugates did not present eye irritation potential (HET-CAM assay). Regarding UVA-induced intracellular ROS evaluation, it was observed that both the tyrosine and tryptophan conjugates showed a slight reduction in UVA-induced ROS production in the monolayer model and did not present any UVA protection in RHS. Thus, it is possible to conclude that mycophenolic acid conjugates are possible candidates for UV filters, especially in the UVB region.

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New data supporting recognition of evident toxicity in acute oral toxicity studies (OECD TG 420)

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Currently there are three Organisation for Economic Co-operation and Development (OECD) test guidelines for acute oral toxicity studies of substances or mixtures. TG 423 and TG 425 use lethality as an endpoint, while TG 420 replaces death with "evident toxicity", defined as clear signs that exposure to a higher dose would result in death. However, the subjective nature of "evident toxicity" may be preventing wider use of TG 420. To address this, the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the European Partnership for Alternative Approaches to Animal Testing (EPAA) collaborated to provide recommendations on the recognition of "evident toxicity". Historical data from acute oral studies were analysed for clinical signs at the lower dose that could have predicted death at the higher dose. Ataxia, laboured respiration, tremors and combinations of signs such as nasal-ocular discharge and/or laboured respiration and/or ataxia and/or tremors are highly predictive. Lethargy, decreased respiration, nasal-ocular discharge and hunched posture have lower but appreciable predictive value. The data has been used to develop recommendations to promote use of TG 420 and thus reduce the suffering and numbers of animals used in acute oral toxicity studies.

Submitted on behalf of the European Partnership for Alternative Approaches to Animal Testing (EPAA) acute oral toxicity task force.

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French Center for 3Rs: Reaching scientists

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In 1959, the ethical 3Rs principle "Replace, Reduce, Refine" was developed by two scientists for scientists. However, 64 years later, it is still a challenge for 3R centers to make a direct impact on researchers with their daily work. The French Ministry of Higher Education and Research and the leading operators of public research created the French Center for 3R (FC3R), which priority is to encourage and implement the 3Rs in France through the promotion of responsible and innovative research, education, and transparent communication by targeting current and future generations of scientists specifically. To do so, the FC3R developed proactive strategies, engaging in concrete actions to federate a synergistic community around the researchers' needs and goals.

The FC3R is working to: 1) disseminate and develop training offers adapted to the specific needs of students and researchers; 2) accompany researchers with their experimental design and the sharing of their unpublished/negative results through the creation of a dedicated platform; 3) communicate in regard to innovative non-animal methods, grants, the evolution of practices and regulations, but also recognize through interviews, conferences and awards stakeholders whose accomplishments lead forward promotion and implementation of the 3Rs in France; 4) fund research projects: two calls were completed in 2022/23 rewarding collaborative initiatives federating the French scientific community around the 3Rs principle and projects promoting Replacement of the use of animals or animal-derived products in science. FC3R will also facilitate scientific collaborations between the academic and industrial research communities, in biology and chemistry.

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Efficient pain relief: Refinement of analgesia for transmitter implantation in mice

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Implementation of telemetry for monitoring of parameters such as ECG, body temperature and activity offers a precise way to measure physiological signals in freely moving animals in preclinical studies. The implantation of transmitters as a harmful surgical intervention itself needs to be refined to minimize impact on further study outcome.

Before and after transmitter implantation mice were treated with different analgesic compounds, administered continuously via drinking water. Animal welfare assessment was carried out by various databased, non-invasive methods such as distress score, burrowing behavior, nesting activity, perianal temperature, mouse grimace scale, water consumption, fecal corticosterone metabolites and telemetric monitoring of ECG, body temperature and activity.

Initial treatment with metamizole and tramadol led to a temporary decrease in bodyweight whereas buprenorphine showed the opposite trend. After implantation of transmitters a lower stress response and an accelerated recovery were observed in buprenorphine treated mice based on the welfare parameters distress score, bodyweight, water consumption, body temperature and activity, when compared to analgesic treatment with tramadol and metamizole.

Buprenorphine as analgesic compound proved to be more efficient in facilitating a sufficient recovery of mice after transmitter implantation, when compared to metamizole and tramadol.

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System animal components-free for application in *in vitro* test toxicology

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In recent years, efforts have been made for ethical, technical, regulatory and economic reasons to reduce or replace the use of animals in testing chemicals and drugs. However, most of the *in vitro* methods used as substitutes were validated containing inputs of animal origin; that is, we cannot consider that they replace 100% the use of animals. We are investigating the adaptation of human adherent cell lines for application *in vitro* toxicity tests based on animal-free chemically defined media. In preliminary analyses, NHK and HaCaT keratinocytes were cultured in medium with and without animal inputs. As a healthy culture control in chemically defined medium and animal product-free, we cultured keratinocytes (HEKa-APF). We evaluated the doubling time. During cultivation in T25 flasks (n = 3), the cell line HEKa-APF reached 100% confluence at 192 h, while the lines NHK and HaCaT were 100% confluent after 336 h. For these strains, the result was similar to cultivation with traditional medium (supplemented with bovine pituitary extract for NHK and supplemented with fetal bovine serum (FBS) for HaCaT) and morphological changes were not perceptible by phase optical microscopy. More in-depth analyzes (viability by MTT, analysis of expression of adhesion molecules, protein profile and ultrastructure) will be performed, with the aim of applying human fibroblasts adapted to OECD guideline 491, at first and then in other standardized methods that still use inputs of animal origin.

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FBS-free conditions for cytotoxicity-based assays: An *in vitro* eye irritation test (OECD TG 491) and acute oral toxicity test (OECD GD 129)

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Fetal bovine serum (FBS) is still being utilized as the universal medium supplement to grow and maintain cells and tissues. However, the use of FBS presents some significant issues such as the animal welfare concerns, batch-to-batch variations and virus contamination risks. Thus, replacing the use of FBS in cell culture media has gained global attention in terms of ethical and regulatory aspects.

In this study, we developed FBS-free conditions for two cytotoxicity-based assays: the Short Time Exposure (STE) test for predicting eye irritation potential and 3T3 Neutral Red Uptake (NRU) cytotoxicity assay for estimating acute oral toxicity. The STE test is a cytotoxicity-based assay that is performed on a confluent monolayer of Statens Seruminstitut Rabbit Cornea cell line, cultured using medium supplemented with 10% FBS. The 3T3 NRU is also a cytotoxicity-based assay that is performed on < 50% confluent monolayer of BALB/c 3T3 mouse fibroblast cell line, cultured using medium supplemented with 10% New borne Calf Serum. We firstly evaluated and compared the performance of two FBS alternatives, human serum (HS) and human platelet lysates (hPLs), with respect to cell morphology and growth rate, and hPLs was selected as a promising alternative to FBS.

We successfully adapted two cytotoxicity-based assays to FBS-free conditions and validated the adapted method using the proficiency substances in OECD TG491 or GD 129. This case study has the potential to be used as a template for transition and evaluation of FBS-free for other cell-based assays.

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Education and training in laboratory animal science and the 3Rs – The contribution of ETPLAS free courses

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The Education & Training Platform for Laboratory Animal Science (www.etplas.eu) aims to improve and harmonize education and training on laboratory animal science and the 3Rs across Europe. ETPLAS issues recommendations, guidelines, and free resources, the most popular being six e-learning courses, which development was sponsored by the EU, and are free to access both in and outside Europe: "Design of Procedures and Projects" (EU-10 and EU-11), "Project Evaluation" (EU-25), "Severity Classification" (EU-12), "Searching for existing non-animal alternatives" (EU-52), and "Developing *In-Vitro* methods" (EU-60). Since their launch, +4500 registered users completed +6000 courses! To facilitate CPD recognition, completion certificates are issued, and estimation of completion time and course contents are available.

Currently, +20 experts are developing 13 new modules, including training core modules "Ethics, animal welfare and the 3Rs, level-1" (EU-2); "Basic and appropriate biology" (EU-3.1) and "Recognition of pain, suffering and distress" (EU-5), the latter two for mouse, rat, zebrafish and four farm animal species. Other include "Ethics, Animal Welfare and the 3Rs, Level-12" (EU-9), "Designated Veterinarian" (EU-24), "Inspectors" (EU-26), and the new module "Competence Assessors", for which learning outcomes have now been developed. A Reflection Group of LAS experts, veterinarians, and National Contact Points provides feedback on scientific accuracy, pedagogical value, and learner experience.

The presentation will cover the new free e-learning modules, which also follow the EU E&T Framework [1], and offer a multimedia-rich, interactive learning experience.

Course providers are welcome to integrate the ETPLAS courses in their teaching, either as stand-alone or reference materials in blended-learning courses.

Reference

[1] https://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/education_training/fr.pdf

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Toxicity testing study of biobased supramolecular lauroyl alanine and derivatives

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Most commercially available surfactants are chemical surfactants that are derived mainly from petroleum products. With rapid advances in biotechnology and increased environmental awareness among consumers, there has been a general desire to find environmentally friendly surfactants that replace petroleum-based surfactants. The products of biological origin, providing a new motivation for the use of superactivity biological surfactants as an alternative. It need to be remindered is toxicology testing should in accordance with the regulations for using alternative methods to animal testing. Lauroyl alanine and its derived salts produced by innovative crafts, offer several advantages, such as low toxicity, high biodegradability and production from renewable materials. In addition, biosurfactants of lauroyl alanyl alanine, lauroyl alanine-arginine, lauroyl alanine-lysine, show greater surface and interfacial activity at low concentrations, and have good activity and stability at different temperature, pH value and salinity.

In the context of the global trend of advocating the gradual replacement of petroleum-based surfactants and the development of green manufacturing and green chemistry industry in China. Under the in influence of the TT21C vision. For the first time, we have completed the first series non-animal toxicity tests of innovative bio-surfactant raw materials in China, proving non-toxic and environmentally friendly, supporting their safe use in laundry, daily chemical, and cosmetic applications.

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Insights from profiling transcription factor transactivation with CYP450 metabolism integration

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Profiling chemical effects on transcription factor activity can help characterize the mechanisms by which chemicals perturb biological systems. Such profiles can contribute to a predictive approach to characterizing chemical effects that avoids animal testing. The Attagene cis-FACTORIAL™ assay uses a reporter system to quantify the activity of 46 transcription factors to provide a quantitative assessment of chemical effects. A new version of this assay, CYP-FACTORIAL™, adds nine key cytochrome P450 (CYP450) enzymes to evaluate effects on transcription factor activity with and without CYP-mediated Phase 1 metabolism. This supports evaluation of whether CYP-mediated oxidation results in an altered bioactivity profile. This study examined activity of 24 chemicals across four test concentrations in the cis-FACTORIAL™ and CYP-FACTORIAL™ assays. Results suggest that alterations in CYP450 metabolism have the greatest effects on transcription factors activating the estrogen receptor (ER), aryl hydrocarbon receptor (AhR), and oxidative stress response (NRF2) pathways. Comparisons of profiles of test vs. reference chemicals identified a highly conserved polycyclic aromatic hydrocarbon toxicity signature involving activation of AhR, NRF2, and ER. Interestingly, a profile in which ER and AhR are activated but NRF2 is not activated correlated to non-toxic compounds, suggesting the possibility of using differences between signatures to predict toxic outcomes. Integrating the profiling approach with metabolism in a multiplexed *in vitro* assay system allows this assay platform to provide insight into chemically induced bioactivity and thus facilitates the development of mechanistically based human-relevant predictive testing approaches.

This project was funded by NIEHS under Contract No. HHSN273201500010C.

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The Latin American Congress on Alternative Methods (COLAMA): Bringing Latin America to alternative methods world

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Alternative methods in Latin America are just a recent issue compared to the European Union and North America. Therefore, to disseminate the theme among Latin American countries, in 2012, the I COLAMA took place in Brazil. As a result of this event, the second COLAMA occurred in Cuba in 2015 and the third in Argentina in 2018. The pandemic impeded the event in 2021, and the IV COLAMA occurred in 2022 in Mexico. All editions received speakers' contributions and attendants from South America, Central America, the Caribbean, North America, and Europe. Industries, universities, and suppliers related to animal experimentation and alternative methods sponsored speakers. COLAMA has also received support from well-known Academic Institutions and non-Government Organizations in Animal Welfare and Alternative Methods such as AALAS, AAALAC International, AWI, CAAT-John Hopkins University, ECVAM, EFSA, FRAME, HSI, InterNICHE, NC3Rs, among others. The four COLAMA editions have had over 200 participants each. COLAMA always focused on issues due to regulatory acceptance, advances in non-animal methods for experimental research, quality assessment and diagnosis, and alternatives in education. Each COLAMA has been the cornerstone to boost the use of alternative methods and set up the 3R's principles in the region. The aim is to perform the congress in different Latin American countries each time so that the fifth edition will take place in Colombia in 2024.

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Analysis of QSAR and antioxidant gene expression for molecular mechanisms based herbal medicine-related hepatotoxicity

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Natural herbal medicines are complexes of various compounds, and it remains difficult to evaluate the toxicity of the active ingredients. Since safety continues to be a major issue with the use of natural herbal medicines, pathway-based cellular stress evaluation is required to evaluate and monitor their toxicity. Therefore, this study is to analyze the toxicity mechanism using the HepG2-TRE-reporter cell lines based on the toxicity mechanism of natural herbal medicines known to induce hepatotoxicity. First, QSAR analysis and molecular docking simulation results of natural herbal medicines (*Xanthium strumarium* L., *Polygala tenuifolia*, and *Ephedra sinica* Stapf) were selected six compounds. We analyzed toxic mechanisms of six compounds and three natural herbal aqueous extracts based on HepG2-reporter cell lines (AP1, P53, Nrf2), which evaluate cellular stress based on imaging analysis. GFP intensity and cytotoxicity were evaluated using high-contents screening after treatment with three natural herbal aqueous extracts and six compounds for 24 and 48 h. In the HepG2-Nrf2 reporter cell line, a significant increase in GFP intensity was confirmed in the treatment of tenuifolin derived from *Polygala tenuifolia* and aqueous extracts and hydroquinone derived from *Xanthium strumarium* L. for 24 h. To confirm the mRNA levels involved in the Nrf2 pathway based on the HCS results, change of NQO1, HMOX1, SRXN1 and SOD1 levels was confirmed. As a result, it was confirmed that the mRNA levels significantly increased. Therefore, our results suggest that HepG2-TRE-reporter cell lines can provide information on molecular mechanisms that initiate cellular stress.

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FBS-free conditions for cell-based assay: An *in vitro* skin sensitization test (OECD TG No. 442D)

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Fetal bovine serum (FBS) is still being utilized as the universal medium supplement to grow and maintain cells and tissues. However, the use of FBS presents some significant issues such as the animal welfare concerns, batch-to-batch variations and virus contamination risks. Thus, replacing the use of FBS in cell culture media has gained global attention in terms of ethical and regulatory aspects.

Skin sensitization cannot be evaluated by a single *in vitro* test and requires several *in vitro* sensitization assays at different stages of sensitization, as outlined in the Adverse Outcome Pathway (AOP) for Skin Sensitization. In this study, we aimed to investigate FBS-free cell culture conditions for one of the *in vitro* skin sensitization assays, KeratinoSens™ that addresses the second key event of the skin sensitization AOP (keratinocytes activation). KeratinoSens™ is an *in vitro* ARE-Nrf2 luciferase test method and generally performed using the medium containing FBS. We evaluated human platelet lysate (hPLs) as a substitute for FBS based on its appropriate growth rate and technical performance by correctly obtaining the expected KeratinoSens™ prediction (EC1.5 and IC50 values) for the 10 proficiency substances recommended in OECD TG442D.

In conclusion, KeratinoSens™ was successfully adapted to FBS-free conditions, and the modified method showed equivalence to the validated reference method as proficiency substances were correctly classified. In further investigations, other *in vitro* skin sensitization tests with FBS-free conditions will be evaluated.

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Reconstructed human intestinal comet assay, an alternative *in vitro* model for genotoxicity assessment

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The aim of this study was to evaluate the compatibility of a reconstructed 3D human small intestinal microtissue model (MatTek EpiIntestinal) to perform the *in vitro* comet assay as a potential higher tier *in vitro* model for genotoxicity. The comet assay is a common follow-up genotoxicity test to confirm or supplement other genotoxicity data. Technically, it can be performed utilising a range of *in vitro* and *in vivo* assay systems. Here, we have suggested a new reconstructed human intestinal comet (RICom) assay protocol for the assessment of orally ingested materials. The human intestine is a major site of food digestion and adsorption, first-pass metabolism as well as an early site of toxicant first contact and thus is a key site for evaluation. Reconstructed intestinal tissues were dosed with eight reference test chemicals: ethyl methanesulfonate (EMS), ethyl nitrosourea (ENU), phenformin hydrochloride (Phen), benzo[a]pyrene (BaP), 1,2-dimethylhydrazine hydrochloride (DMH), etoposide (Etop), Potassium Bromate (KBr) and glycidamide (GA) over a span of 48 hours. The RICom assay correctly identified the genotoxins EMS, ENU, KBr and GA. Phen HCl, a known non-genotoxin, did not induce DNA damage in the 3D reconstructed intestinal tissues whilst showing high cytotoxicity as assessed by the assay. The 3D reconstructed intestinal tissues possess sufficient metabolic competency for the successful detection of pro-genotoxicity elicited by BaP, without the use of exogenous metabolic system. In contrast, DMH, a chemical that requires liver metabolism to exert genotoxicity, did not induce detectable DNA damage in the 3D reconstructed intestinal tissue system.

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Development of a defined approach for eye hazard identification of neat solids according to the three UN GHS categories

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Currently two OECD adopted defined approaches (DAs) for Serious Eye Damage and Eye Irritation for non-surfactant liquids exist (OECD TG 467, 2022). The purpose of the current study was to enlarge the applicability domain to solids with a new DA. The DA for neat solids (DAS) combines test methods described in OECD TG 437 and TG 492.

The DA is developed based on in-depth statistical analysis of a database on solids for which *in vitro* and historically curated *in vivo* Draize eye test data exist. The performance of the DAS was assessed by comparing the prediction results with the UN GHS classification (based on historical *in vivo* data).

In a first tier of the DA, the SkinEthic™ HCE EIT method (TG 492) is used to distinguish No Cat. from classified substances. For classified substances the BCOP LLBO method (TG 437) is used to identify Cat. 1, the remaining solids are then predicted Cat. 2.

In summary, 73.2% Cat. 1 (N = 28), 52.3% Cat. 2 (N = 18) and 70.0% of No Cat. (N = 60) solids were correctly identified, giving a balanced accuracy of 65.2%. The percentage of correct predictions was close to the criteria set by the OECD Expert Group for Skin/Eye Irritation and Phototoxicity (75% Cat. 1, 50% Cat. 2 and 70% No Cat.) for non-surfactant liquids. The percentage of mispredictions was below the established maximum values. The DAS has shown to be successful for eye hazard identification of solids according to the three UN GHS categories.

Presentation: Poster

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Prediction of the skin sensitisation potential of vanillin using read-across in NGRA

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Skin sensitisation is a key adverse human health effect to be addressed in the safety assessment of cosmetic ingredients. Regulatory demands and scientific progress have led to the development of a Next Generation Risk Assessment (NGRA) framework, relying on the use of New Approach Methodologies (NAM), Defined Approaches (DA) and read-across instead of animal models.

This case study illustrates the application of read-across for the prediction of the skin sensitisation potential of vanillin at the hypothetical use concentration of 0.5% in two exposure scenarios, shower gel and face cream.

A stepwise process was applied to select the most suitable analogues based on protein reactivity, structural characteristics, physicochemical properties, skin metabolism profile and availability of skin sensitisation data.

The initial set of seven identified chemicals with sufficient skin sensitization data ranging from strong to non-sensitizer were further narrowed to four weak or non-sensitizer chemicals based on skin metabolism consideration.

The applied read-across approach predicted a weak skin sensitiser potential for vanillin corresponding with a Local Lymph Node Assay EC3 value of 10%. Based on this EC3 value a point of departure of 2500 µg/cm^2 was derived, resulting in an acceptable exposure level (AEL) of 25 µg/cm^2. Because the consumer exposure levels (CEL) for the face cream (13.5 µg/cm^2) and shower gel (0.05 µg/cm^2) scenarios were lower than the AEL, the NGRA concluded both uses as safe.

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Distress analysis for evaluating the toxicity of traditional Chinese medicine

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Purpose: Traditional Chinese medicine, bufalin, exhibits great potential on antitumor therapy and also severe cardiotoxicity, which limits its further clinical application. The purpose of this study was to apply the advanced method (animal stress analysis, nesting behavior) to investigate the toxicity of bufalin, comparation with electrocardiogram and blood biochemical index.

Methods: After injection of bufalin and cancer cell membrane camouflaged bufalin with different dosage into mice, the wellbeing of these animals was evaluated by assessing nesting behavior, body weight and a distress score. Also, the electrocardiogram was recorded, and the blood was collected to test biochemical index.

Results: Mice after injecting with 1mg/kg bufalin had a higher survival rate, and their biochemical index such as alanine transaminase (ALT), aspartate aminotransferase (AST), creatinine almost didn't change. However, their wellbeing and behavior significantly changed. Fortunately, after we encapsulated bufalin with cancer cell membrane and given it to mice, their wellbeing was significantly better.

Conclusion: Animal stress analysis and nesting behavior seem more sensitive and more direct to observe, when evaluating the acute toxicity of bufalin. It shows great potential to extend its application to pharmaceutics, biomedicine, etc.

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SkinEthic™ HCE time-to-toxicity OECD adopted method as an alternate of the *in vivo* standard for surfactants?

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Historically, *in vivo* Draize data has been used as the reference database to evaluate *in vitro* test methods for eye hazard identification. From current ethical practice, it is difficult to add more substances to the reference database for the evaluation of a defined approach (DA) based on the. This is especially true when considering the assessment of a DA for surfactants as no additional surfactants with qualified *in vivo* data have been identified.

The current reference database for the DA on surfactants contains 49 substances, of which 23 are UN GHS Eye Hazard Category 1 (Cat. 1), 9 Cat. 2 and 17 substances that do not require UN GHS classification (No Cat.). The purpose of the current study was to evaluate these surfactants using the only stand-alone test method, namely SkinEthic™ HCE Time To Toxicity, which was adopted to predict all three UN GHS categories (OECD TG 492B, 2022). The performance was assessed by comparing the results with the historical *in vivo* data classification. The prediction reached the minimum performance values of 75% Cat. 1, 50% Cat. 2, and 70% No Cat. established by the OECD expert group. The SkinEthic™ HCE Time-to-Toxicity test method was also proposed to generate data on additional surfactants that can be used to evaluate the performance of the DA on surfactants.

In conclusion, considering the SkinEthic™ HCE Time To Toxicity as an alternate of *in vivo* standard opens the possibility for progress in phasing out animal testing and may pave the ways for other endpoints.

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Ocular assessment of challenging chemicals using the OECD TG492B SkinEthic™ HCE time-to-toxicity adopted method

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The SkinEthic™ HCE Time-to-Toxicity test method is the only stand-alone NAM adopted by OECD for discriminating the three UN GHS ocular hazard categories (OECD TG 492B, 2022).

The test method evaluates the hazard potential of a chemical based on its ability to induce cytotoxicity. The method consists of 2 protocols (for liquids and solids). Based on the viability observed for the different exposure periods (from 5 to 120-min) a classification is assigned.

The within laboratory reproducibility from the 3 laboratories was 90% for liquids and 100% for solids while the between laboratory reproducibility was 80% and 100%, respectively. When considering all 151 chemicals, the test method has a balanced accuracy of 74% with correct predictions of 79% for Cat 1, 69% for Cat 2 and 75% for No Cat, when compared to reference *in vivo* rabbit eye test data.

In order to illustrate the relevance of the test method on challenging chemicals, an evaluation of 10 standards and 10 direct MTT-reducers ± colour interfering chemicals was performed. Amongst those, 4 chemicals were incompatible with use of standard photometry detection system. Determination of the cell viability leading to determination of classification was achieved by High-Performance Liquid Chromatography (HPLC)-spectrophotometry. Overall, these results highlighted the relevance of the HPLC-spectrophotometry detection system, independently of the protocols being performed.

In conclusion, the SkinEthic™ HCE Time To Toxicity could be recommended as a full replacement to the *in vivo* Draize acute eye irritation test for classification of these challenging substances.

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Acceptance of NAMs in safety assessment: Innovation science meets toxicology

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The implementation of 3Rs for human health safety assessment of chemicals is a tedious process in which New Approach Methodologies (NAMs) slowly proceed from the R&D phase towards acceptance and implementation into regulatory frameworks. As a result, only for simple toxicological endpoints, such as genotoxicity and eye and skin irritation, validated *in vitro* tests have been implemented. For more complex endpoints, such as repeated dose toxicity, no NAM-based test guidelines are currently available. In the Virtual Human Platform for Safety Assessment project (www.vhp4safety.nl, NWA-ORC 1292.19.272), NAMs are integrated with Adverse Outcome Pathways (AOPs) and data science to investigate whether safety can be predicted for complex human health effects. This integrated approach provides an even greater challenge towards acceptance and implementation in regulatory frameworks since uncertainties are introduced at multiple levels. In this study, toxicologists and innovation scientists collaborated to develop a mission-oriented innovation system (MIS) framework. The framework describes key processes that are important for regulatory acceptance and implementation of NAMs as well as actors that are involved in these processes. Examples of such key processes are R&D activities, validation and social legitimacy. The framework looks beyond solely technological problems and takes into account socio-institutional aspects that need to be changed and fulfilled in order for a systemic change to occur. As such, the framework helps to identify drivers and barriers for NAM acceptance. This focus on systemic change makes the MIS approach a promising tool for the mission: to achieve reliable safety testing of chemicals based on NAMs.

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Set up and validation of behavioral analysis for cannabinoids assessment in zebrafish

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Zebrafish is a powerful alternative model allowing cost-effective *in vivo* screenings, following the 3Rs principles when working with larvae below 5 days post-fertilization. Advantageously, full behavior analysis could be evaluated in zebrafish larvae at this stage. While the Light-Dark Transition test is currently used to identify neuroactive substances, sleeping and anxiety assays are not well-established yet for Drug screening. Since these assays would be precious in cannabinoids screening seeking new therapies for anxiety, sleep disorders, and other neurobehavioral affections are set up and validated.

This project aims to validate the Light-Dark TransitionTest, Anxiety Assay, and Sleeping Assay with well-known reference compounds, determining the most relevant endpoints for the evaluation of cannabinoid effects in each one.

5 concentrations of diazepam, caffeine, PTZ and yohimbine were tested as reference compounds. Afterwards, 4 cannabinoids were assessed to evaluate the suitability of the established behavioural assays for their classification.

As a result, total distance moved, distance moved in the periphery/total distance and sleep cumulative duration were chosen as main endpoints in the Light-Dark Transition Test, Thigmotaxis, and Sleeping Assays, respectively. The reference compounds were classified as expected: Diazepam as hypoactive, anxiolytic, and sleep-inducing; Caffeine as hyperactive; PTZ as the most suitable anxiogenic reference and Yohimbine as the best sleep-depriving compound. The 3 neurobehavioral assays are suitable for cannabinoids screening, accurately classifying 4 tested cannabinoids as hypoactive, anxiolytic, and sleep-inducers.

In brief, these novel neurobehavioral assays are successfully set up and validated for the cannabinoids screening in the zebrafish alternative *in vivo* model.

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Virtual human platform for safety assessment (VHP4Safety)

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The VHP4Safety project (www.vhp4safety.nl, NWA-ORC 1292.19.272) aims to protect human health and to improve the safety assessment of chemicals and pharmaceuticals without the use of animal models. Three case studies are designed to incorporate human relevant scenarios to discriminate vulnerable groups that are not addressed with animal studies. These include disease state (kidney failure) upon exposure to pharmaceuticals, life course exposure to pesticides and neurodegeneration (Parkinson's disease), and sex and age differences upon exposure to chemical substances in the development of thyroid-mediated brain development. Several data sources, e.g., data on human physiology, chemical characteristics, existing (omics) data, epidemiology data, toxicokinetic and -dynamic parameters, are integrated within Adverse Outcome Pathway (AOP) networks together with data generated from *in vitro* assays representing key events in the AOPs. These data feed into the VHP4Safety *in silico* platform built on tools and services such as PBPK and Artificial Intelligence models. We will present the first contours of the VHP4Safety platform, the case study workflows that feed the platform and the activities towards the implementation of the platform, such as setting performance criteria, regulatory acceptance and education and training. Through active involvement of academic, regulatory, industrial and societal partners covering the entire safety assessment knowledge chain, we are developing a new framework to determine the safety of chemicals and pharmaceuticals to protect human health solely based on human biology. While working together with other initiatives, such as the European projects of the ASPIS cluster, VHP4Safety will accelerate the transition to animal-free safety assessment.

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Characterization of respiratory sensitizing properties of the protein subtilisin using GARDair

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Sensitization is a disease state induced by immune system response to certain proteins or chemicals, referred to as sensitizers. Proactive identification of sensitizers is central in hazard and risk assessment of both biologics and chemicals, for regulatory registration or to ensure occupational safety. While large investments in New Approach Methodologies for assessment of dermal sensitizers have been made, the ability to accurately predict respiratory sensitizers *in vitro*, both biologics and chemicals, remains unfulfilled.

The Genomic Allergen Rapid Detection assay for assessment of respiratory sensitizers (GARDair) is based on the same framework as the OECD validated GARDskin (OECD TG 442E) assay, providing binary hazard identification of chemical respiratory sensitizers via transcriptional changes induced by the TSLP-receptor signaling pathway and immune response polarization towards a Th2 response. In this study, we hypothesized similar toxicity pathway engagement by protein sensitizers with the use of GARDair technology and potential application for identification of protein allergens. To test this hypothesis, standard GARDair assay protocols were implemented as well as the use of the well-characterized protein allergen Subtilisin as a positive control. Preliminary data have validated Subtilisin to activate pathways associated with the TSLP receptor as well as accurately identified it as a respiratory sensitizer.

Based on these preliminary findings, the assay has been identified as fit-for-purpose for prediction of chemical and protein respiratory sensitizers. These findings constitute a promising path forward towards the implementation of a standardized tool in production and product development settings to promote occupational safety using animal-free methods.

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Validation of clinical scoring and home-cage monitoring in a mouse model of acute colitis

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For ethical and legal reasons, it is necessary to assess the welfare of experimental animals as well as possible. The aim of this study was to evaluate the benefit of a home cage monitoring system for improved welfare assessment during dextran sulfate sodium (DSS)-induced colitis. Additionally, potential impacts on activity patterns due to handling procedures using tunnel or tail handling were analyzed.

For welfare assessment, animals were clinically scored using a clinical score sheet and an automated home-cage monitoring system was used for continuous activity monitoring.

Female C57BL/6J mice were exposed to graded DSS doses via drinking water for colitis induction. The general appearance, body weight, stool consistency, fecal occult blood (FOB) and home cage activity were daily assessed for the monitoring of the general health status and behavioral changes of the animals.

DSS-treated mice showed increased clinical scores and positive FOB tests when compared to control mice. Moreover, a significant decrease in activity and body weight due to a higher dosage of DSS administration was detected. Activity heat maps showed differences in activity patterns between DSS-treated mice and control groups. Furthermore, no significant differences between tunnel and tail handled mice cohorts methods were detected. A binary classifier of correlated body weight and activity data enabled the classification of severity related to treatment.

Home-cage monitoring enabled welfare assessment in a DSS-induced colitis model equally well as gold standard clinical parameters. In addition, it revealed changes in activity patterns due to routine handling procedures applied in experimental model work.

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Developmental neurotoxicity induced by glutaraldehyde in neuron/astrocytic co-cultured cells and zebrafish

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Developmental neurotoxicity induced by Glutaraldehyde (GA) was assessed on SH-SY5Y/Astrocyte co-cultured cells and zebrafish. GA exposure inhibited neurite outgrowth at the cells. GA exposure decreased survival and induced myelination defects in oligodendrocytes of zebrafish.

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Advanced visualization using a transgenic zebrafish for pyrethroid insecticide neurotoxicity

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The purpose of this study was to investigate the neurological effect of pyrethroid insecticides on zebrafish. Pyrethroid insecticides are known to be toxic to aquatic invertebrates and affect the nervous system in insects. Allethrin, one of the pyrethroid insecticides, is classified as an endocrine disrupting substance and is concerned about health effects. In this study, the neurotoxicity of 7 pyrethroid insecticides (Imiprothrin, Tetramethrin, Phenothrin, Prallethrin, Allethrine, Zeta cypermethrin, Permethrin, etc.) was analyzed using the zebrafish model.

Neurotoxicity was evaluated using transgenic models (Tg(sox-10:eGFP), Tg(elavl3:eGFP), Tg(mbp:mGFP)) in which signals from the nervous system were observed. The LC25 value for each substance was obtained through a preliminary experiment, and neurotoxicity analysis was performed on the concentration of the LC25 value. As a result, when compared with the control group, it was confirmed that the width of the brain and spine of the central nervous system decreased and development was reduced, and abnormalities were found in the neuron shape of the nerve crest cells in the spinal cord. It was also confirmed that the development of

myelinating oligodendrocytes and Schwann cells and brain fluorescence intensity were reduced.

In terms of regenerative capacity, the spinal cord is an important neurotoxicity biomarker. And myelination disruption may play an important role as an indicator for diseases such as multiple sclerosis and hereditary myelin disease. In this study, we confirmed that 7 Pyrethroid insecticides exhibit neurotoxicity.

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Patient-derived lung tissue represents an alternative model for the development of anti-fibrotic drugs

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Pulmonary fibrosis (PF) is a severe lung disease, causing irreversible dysfunction of the organ. Current animal models often do not represent the exact clinical picture of the disease and are therefore of limited relevance. With the use of Precision-Cut Lung Slices (PCLS), and a general shift from *in vivo* to *ex vivo* models, the aim was to establish a novel alternative human based 3R model for the development of anti-fibrotic drugs.

PCLS prepared from non-fibrotic tissue sections (lung cancer patients) triggered with a pro-fibrotic cytokine cocktail (TGF-β/TNF-α) revealed significantly elevated RNA as well as protein levels of several integrins (ITGAV, ITGB6), extracellular matrix (ECM) remodeling enzymes (e.g., metalloproteinases), cytokines (IL-1β, IL6) and relevant mediators involved in the initial wound repair mechanism and early fibrotic response (Plasminogen-activator-inhibitor-1). PCLS prepared from PF patients showed > 5000 significantly up- and down-regulated genes after 24 h cultivation as compared to non-fibrotic PCLS. Gene set enrichment analysis identified 5 significantly distinct genetic patterns in PCLS from non-fibrotic vs. fibrotic slices, including molecular pathways of p53- and FoxO signaling and fatty acid, carbon or purine metabolism. Supernatants of these PCLS revealed higher levels of FN1 (2-fold), pCol1a1 (5-fold) and elevated alpha-smooth-muscle-actin levels in the tissue as compared to non-fibrotic PCLS. Most importantly, in both experimental set-ups, the efficacy of an anti-fibrotic reference treatment could be confirmed.

Overall, this alternative *ex vivo* method has great potential to investigate important signaling pathways and general pathomechanisms of early-onset- or end-stage-fibrosis without using animals to drive the development of new anti-fibrotic drugs.

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Molecular mechanisms based on liver toxicity of metyrapone toward zebrafish

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Drug-induced liver injury (DILI) is a major issue in clinical medicine and drug development. Zebrafish is attracting attention as a model for predicting it. Zebrafish share 84% of the genes associated with human disease. Additionally, zebrafish can be a strength in DILI studies due to their rapid development at the embryonic stage, and high productivity combined with optical clarity. Nonalcoholic fatty liver disease (NAFLD), characterized by the accumulation of excess fat in liver cells, has increased in prevalence in recent years. NAFLD is closely associated with several side effects, including obesity, insulin resistance, type 2 diabetes, and cardiovascular disease. Cushing's syndrome, caused by prolonged exposure to high levels of cortisol, has been reported to be often associated with NAFLD. In this study, we investigated the effect of exposure to the commercialized drug Methyrapone (MTR) on embryogenesis in zebrafish. MTR is used for the medical control of high cortisol in Cushing's syndrome. It works by inhibiting the production of adrenal steroids. Although no clinical disease has been reported in the liver for MTR, in our study, MTR was found to reduce translucency in the early developmental liver of zebrafish. Then, fatty liver was induced and confirmed by Oil red O staining. To determine the effect on gene expression, Quantitative Real time Polymerase chain reaction (qRT-PCR) was performed. In this regard, we investigated genes involved in lipid metabolism, ER stress, apoptosis and inflammation. It was confirmed that the number of neutrophils and macrophages was reduced using the transgenic zebrafish.

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Life course pesticide exposure and Parkinson's disease: Innovative animal-free assessment of chemical effects on neurodegeneration

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Exposure to pesticides has been associated with an increased risk of developing Parkinson's disease (PD). Studies following OECD Testing Guidelines are not aimed at, and thus incapable of capturing, indications of neurodegeneration. Moreover, late hallmarks of human neurodegeneration cannot be replicated in rodents. As such, it is questionable to what extent rodent studies provide sufficient insights into effects that may lead to disease later in life. Therefore, there is a need to improve the assessment strategy and prediction of potential harmful effects of chemicals related to neurodegeneration.

To create an alternative workflow, this case study was included in the Virtual Human Platform for Safety Assessment (VHP4Safety) project. The case study is firmly rooted in charting the human physiology underlying development of PD and applying the adverse outcome pathway (AOP) framework. To quantify AOP networks, concentration-response and response-response relationships will be determined using a dedicated *in vitro* test battery. Integration of these data will allow for extrapolation of changes along linked key events, leading to a prediction on the role of chemicals in the occurrence of PD in humans. This should ultimately be integrated in a virtual human approach suitable for regulatory safety and risk assessment of chemicals.

Within this project, we aspire to 1) provide the biological basis of human PD development needed for constructing the virtual human as part of VHP4Safety and 2) establish a novel mechanism-based and animal free approach to test neurodegenerative properties of compounds, enabling inclusion of neurodegeneration in regulatory safety and risk assessment guidelines.

Presentation: Poster

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Going the extra mile for NAM acceptance: Engaging stakeholders for next generation risk assessment

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ONTOX will deliver a strategy to create innovative new approach methodologies (NAMs) in order to predict systemic repeated dose toxicity effects of any type of chemical that, upon combination with tailored exposure assessment, will enable human risk assessment. The project focuses on six specific NAMs addressing adversities in the liver, kidneys and developing brain. The NAMs will consist of an ontology-driven and artificial intelligence-based computational system, fed by available physiological human data and targeted *vitro* and *in silico* testing.

To ensure implementation in risk assessment practice and to maximise end-user acceptance and regulatory confidence, NAMs will be developed, evaluated and applied in collaboration with the most relevant stakeholders throughout the project. For this reason, industry from different sectors, NGOs, policymakers and regulatory authorities were interviewed. Secondly a survey with questions related to the acceptance and performance criteria, validation and user requirements was carried out to identify stakeholders' position in relation to NAMs and probabilistic risk assessment. The results of this survey (27 responders) demonstrated the differences between and within the stakeholder groups. Thirdly, a selection of debatable topics, were discussed in a stakeholder network meeting. The outcome of the survey and the stakeholder network meeting will be presented. For those challenges that cannot be solved with the current stakeholders, we will organise a hackathon with a mixed group of participants. The hackathon participants will exist of people within and from outside the risk assessment domain. This enables an out-of-the-box approach to solve tough challenges and make use of interdisciplinary expertise.

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Today's students are tomorrow's researchers

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The Swedish National Committee and its executive body, the Swedish 3Rs Center, are using several different approaches to reach out to students, both at younger and older ages. Through these initiatives we increase knowledge and understanding of animal experimentation as well as different types of animal-free methods.

We have produced a digital educational material for the upper secondary school. The material is well-connected to the curriculum and concerns research, animal experimentation, ethics and the 3Rs. Together with the material, there is a clearly written teachers guide. During the lectures, students gain new knowledge through text, images and short films. There are also active learning sessions with questions and discussion. In connection to this material we have organised a writing competition encouraging students to imagine what a world without animal experimentation could look like and depict this in a short story or a poem.

To further reach out to schools we participate in Researchers' Night, a yearly science festival in the EU. Each year we bring researchers to meet students and talk about the 3Rs and related subjects.

We also offer specific educational activities for future animal technicians. During these sessions we focus on non-aversive handling of mice and rats and on existing and future efforts to replace animal experimentation.

By exposing students to perspectives on animal experimentation and the 3Rs already at a young age, we aim to foster a non-polarized dialog based on the premises of always choosing the best suited research method available.

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NAMs: Target image immunology

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To facilitate the transition towards the use of animal free innovations, the Netherlands National Committee for the protection of animals used for scientific purposes has invited researchers to write target images for their field of work. We here present the target image for the field of Immunology.

Immunological research is aimed at understanding molecular and cellular processes that underlie pathologies of human diseases as well as infections, and to develop interventions to treat or prevent these diseases and infections. Animal models have been, and are still being, used to model many of these processes. However, it is becoming increasingly clear that animal models are indeed models and importantly, they might not even be the best models. The field of immunology moves very fast and new technological and medical advances have led to the development of innovative non-animal, or new approach methods (NAMs) with the potential to replace animal models.

We have chosen to first identify the types of NAMs that are currently being developed and/or used in immunology research. We then analyzed the benefits but also the limitations of NAMs and have used this to develop targets for the near and for the not-so-near future. Finally, we have identified the most important barriers for implementation of NAMs and discuss how these can be addressed. Specific recommendations for the development and implementation of NAMs in the field of immunology will be presented. These are aimed at improving science while at the same time reducing our scientific dependance on animals.

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Improved post-operative pain and welfare assessment in sheep

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In orthopedic translational research, good pain management is mandatory for good animal welfare and methods for adequate pain and welfare assessment are rare. In search for an improved pain recognition, the aim of this study was the evaluation of facial expressions and telemetry-derived measurements after surgical interventions in sheep.

After unilateral tibia osteotomy or subcutaneous transmitter implantation, followed by unilateral tendon ablation in German black headed mutton ewes, clinical scoring was compared to SGS or the telemetry-derived parameters heart rate, temperature and activity. For the analysis of facial expressions, video recordings were performed and blinded observers retrospectively assessed the Sheep Grimace Scale (SGS). In addition, saliva samples were collected and cortisol levels were determined using an enzyme-linked immunosorbent assay.

After transmitter implantation and tendon ablation, clinical score and heart rates were slightly increased, whereas activity was decreased in individual sheep, especially within the first day after surgery. Body temperatures and cortisol levels were not altered. Analysis of the video footage revealed significantly elevated SGS after tendon ablation within the first two days or for up to one week after osteotomy. In contrast, SGS only tended to be increased after transmitter implantation.

In conclusion, SGS and telemetry-derived heart rate and activity indicated pain after the surgical interventions. Therefore, these methods, especially the non-invasive SGS, are highly recommended as additional parameter to clinical scoring for optimal pain recognition and postoperative management in sheep, consequently contributing to refinement.

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The 3Rs Centre Utrecht: Facilitating behavioral change by researchers

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The 3Rs Centre Utrecht (3RCU) is located centrally in the Netherlands in one the largest biomedical research hubs of the country, the Utrecht Science Park. Our mission is to stimulate the development, acceptance and implementation of methods that can Replace, Reduce and Refine animal experiments. In that order.

We host a website that is a central hub for the 3Rs containing useful public tools and databases such as the humane endpoints website and the fetal calf serum-free cell culture database. We also communicate on recent 3Rs developments via social media as Linkedin and Twitter.

The 3RCU is part of a larger Utrecht initiative aiming to do better science with fewer animals. We are embedded within the Animal Welfare Body Utrecht, that supervises the welfare of laboratory animals and the quality of animal experiments in research and education, and we are closely affiliated to the TPI Utrecht program (Transition Program towards animal-free Innovation).

This embedding provides us with direct access to researchers that are using, or that are planning to use, experimental animals. We aim to maximally facilitate researchers when they are considering *in vitro* methodology instead of *in vivo* approaches. Our strategy encompasses the use of targeted campaigns via social media to create adherence, the organization of working groups, internships, centralization of resources and bench space to create data in addition to ideas. We will present one program to showcase this integral approach making the 3RCU unique in the diversity of 3R Centres.

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Prioritizing in the replacement of animal models

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The Netherlands National Committee for the protection of animals used for scientific purposes (NCad) investigated which animal models should preferably be replaced by New Approach Methodologies (NAMs). The idea was to arrive at a set of criteria pointing to the ethically most problematic animal models and experiments, for which replacement is therefore needed.

A short list of eight criteria was analysed more in depth. These were proportionality, the stage of development of non-animal alternatives, the number of animals used, the animal species, translatability, the relevance of the aim of the experiment, the level of suffering and the level of infringement of integrity.

Based on the analysis, six criteria were considered useful. The stage of development of a NAM is relevant as this determines the feasibility or resources needed to replace the animal study. The number of times an animal model is used and thereby the number of animals used in the long run should also be considered a criterium. The relevance of the aim of the study and the level of suffering are components of the harm-benefit analysis already. Most innovative is that we propose to use the infringement of the integrity of an animal and translatability as explicit criteria to prioritize in replacing animal models and experiments. Together, this set of criteria can be helpful to actors like financiers and regulators to work towards the goal of replacing animal models and experiments, starting with those that are ethically the most problematic. These criteria can also enrich the harm-benefit analysis.

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The 3Rs principle and the validity of scientific research – A guideline by the Permanent Senate Commission on Animal Protection and Experimentation of the German Research Foundation

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The right to freedom of research as enshrined in the Basic Law for the Federal Republic of Germany and the constitutional objective of animal welfare are two important values with high relevance to animal experimentation in research in Germany. The "Permanent Senate Commission on Animal Protection and Experimentation of the German Research Foundation" is an interdisciplinary Committee of Experts that aims to mediate between these two values and striking a balance to achieve the best outcome for both interests in regards of the use of animal in research. To this end the Senate Commission published their guideline "Animal Experimentation in Research: The 3Rs Principle and the Validity of Scientific Research" on design and description of animal research projects. The effort to maximise the scientific validity and replicability of research findings while observing animal welfare through adhering to the 3R principle must be the basis of study designs in animal research. In practice, tensions may arise between measures taken to advance animal welfare and the requirements for ensuring scientific validity. Because of the interdependence of these aspects, policies for implementing the 3Rs principle should not be considered in isolation but should rather be integrated in the study design and included in descriptions of research projects. With these guidelines, the Senate Commission aims to contribute to the debate on quality in biomedical research and to help define the specific requirements for conducting animal experiments. In addition, this publication supports researchers in the design and adequate description of research projects involving animal experimentation.

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On the usefulness of animals as a model system: A rational framework to address an emotional discussion

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Banning or reduction of the use of animals for laboratory experiments is a frequently-discussed societal and scientific issue. Moreover, the usefulness of animals needs to be considered in any decision process on the permission of specific animal studies. This complex issue is often simplified and generalized in the media around the question, "Are animals useful as a model?"

The series of articles named "On the usefulness of animals as a model system" – and published in the ALTEX journal Benchmark corner – has been initiated to provide a neutral platform for a rational discussion addressing this question and providing tools to determine the value of using animal and non-animal models in different application areas.

To render this often-emotional discussion about animal experimentation more rational, it is important to define "usefulness" in a structured and transparent way. This series of articles discusses the overall benchmarking process by specifying six issues: (i) consistency of animal-derived data (robustness of the model system); (ii) scientific domain investigated; (iii) measurement unit for "benefit" (integrating positive and negative aspects); (iv) benchmarking to alternatives; (v) definition of success criteria (how good is good enough); (vi) the procedure to assess benefit and necessity.

The goal is to guide what needs to be clarified in scientific and political discussions. This framework should help in the future to structure available information, to identify and fill information gaps, and to arrive at rational decisions in various sub-fields of animal use.

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Counting down – What do animal statistics tell us about the 3Rs and animal welfare in research?

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In recent years thorough statistics on the use of animals in research has been one tool to promote transparency in research with animals. Across the EU, member states publish their statistics on the use of animals in research annually and the EU Commission launched their database "ALURES" in 2021, which provides a comprehensive overview of these statistics to inform the public. In the public discussion on animal research these statistics have become a focus point and a synonym for good or bad welfare of laboratory animals. While aiming on replacing animal experiments with sufficient non-animal methods and reducing the number of animals needed in experiments should be a key goal in animal research and testing, it shouldn't be the main motivation that drives the discourse, nor the main indicator of progress as the statistics can't capture important aspects such as efforts of refinement, improvements of experimental methods or the scientific findings achieved through animal research. The use of animals in research is complex, with complex reasons, requirements and responsibilities and reducing it to one statistic veils the actual complexity of the topic to the public. While having a thorough statistic on the use of animals in research is certainly useful and informative, it needs to be embedded within a broader narrative and information to reflect the complexity of the subject and communicate it to the public.

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The Swedish 3Rs Center – Five years of focus on the 3Rs

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The Swedish 3Rs Center is located at the Swedish Board of Agriculture and has the Swedish National Committee for the Protection of Animals Used for Scientific Purposes as its steering group. The 3Rs Center has just recently celebrated its first five years and it is today a natural contact point for the 3Rs in Sweden.

An important cornerstone in the Center's work is that it includes all 3 Rs – Replacement, Reduction and Refinement. The target groups thus also range all the way from researchers, funding- and governmental agencies to animal technicians and veterinarians and further to politicians and pupils.

The 3R Center's activities can be divided into several different areas: communication and networking, seminars and meetings, recommendations, education, information materials and supporting activities. Some mentionable examples are a digital focus letter, annual meetings for animal welfare bodies and funding agencies, recommendations on group housing of male mice and marking and tagging of fish, digital educational material for the upper secondary school, infographics on evaluation and formal validation of animal-free methods and collaboration with other Swedish government agencies.

Working with all 3Rs is a strength when reaching out to our target groups. Information on Replacement and Refinement can be combined in the same activities and reach a broader audience. This inclusive approach is also true for the Swedish National Committee which is composed of representatives from academia, industry and animal welfare organizations. Thus, many different aspects of the 3Rs are represented and allowed to permeate the organization and our activities.

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Assessment of postoperative pain in rats under analgesia with PET imaging

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Quantification and correct localization of pain are important aspects of animal welfare assessment. However, severity tests are often not sensitive enough to accurately detect pain and under analgesia it is difficult to assess whether the animal would suffer from pain without medication. Following the need to sensitively assess pain, the aim of this study was to objectively quantify and localize postoperative σ1-receptor-mediated pain during and after analgesia.

Eighteen rats underwent surgical interventions (skin-incision or partial liver resection), while nine rats served as control. Analgesia was administered for three days consecutively. PET/CT imaging was performed with [18F]Fluspidine on days 1, 4, and 7 after surgery. At each time point, three animals were euthanized for histological assessment of incision sites and organs. Postoperative pain was also monitored by score-sheet, Open-Field-Test, and Von-Frey-Test.

Despite analgesic treatment, image analysis and immunohistochemistry showed a significantly higher σ1-receptor expression on postoperative day 1 at the incision site of rats that underwent partial liver resection compared to the other groups, which might be caused by the additional opening of the peritoneum. However, behavioral and pain tests showed no impairment of animal welfare due to efficient analgesia. No σ1-receptor-mediated pain could be detected by image analysis and histology on days 4 and 7, which is in line with the Open-Field-Test and score-sheets.

Imaging was able to sensitively detect postoperative σ1-receptor-mediated pain independent of analgesia administration. Therefore, [18F]Fluspidine PET/CT imaging may remarkably refine pain monitoring in future preclinical studies and may be used as reference to other severity tests.

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All animals count – Emergence of surplus animals has to be reduced globally

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In 2023, all European member states have to report again the number of animals that have been bred but not used for scientific purposes to the European Commission. The first report for 2017 has shown that with about 12.6 million animals this number significantly exceeds the number of 9.4 million animals that were used in experiments. These so-called "surplus" animals regularly derive from breeding genetically altered animals, when i.e. animals do not exhibit the required genetic changes and are thus killed without being used in a procedure.

In Germany, it is legally forbidden to kill any animal without a reasonable cause. Violations can be punished by fines or imprisonment for up to three years. Recently, the German Federal Administrative court decided to ban the killing of male chicken immediately after hatching as it saw no justification for it. This judgement has encouraged German NGOs to file criminal charges against several laboratory animal facilities accusing them of killing "surplus" animals without a reasonable cause.

Even though the concept of the reasonable cause might be a German peculiarity, "surplus" animals are a global phenomenon. Minimizing the number of "surplus" animals has the greatest potential to reduce the number of laboratory animals. Optimized breeding strategies and colony management, use of cryoconservation, gene editing methods and animals of both sexes are some measures to reduce "surplus" animals. As a first step the number of "surplus" animals should be transparently and annually reported in every country. From 2021 onwards, Germany has decided to do so.

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The GOLIATH project on metabolism disrupting compounds: Test methods assessment

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GOLIATH, one of eight projects focused on endocrine disrupting chemicals within the EU H2020 EURION cluster, was set-up to address the lack of new approach methodologies (NAM) for chemicals that disrupt metabolic processes – collectively referred to as metabolic-disrupting chemicals (MDCs). MDCs are natural or anthropogenic chemicals promoting metabolic changes that can ultimately contribute to obesity, diabetes and/or fatty liver in humans. However, MDCs are not yet addressed by chemical regulations.

Focussing on the main cellular targets of metabolic disruption, GOLIATH is developing, optimising, and pre-validating NAMs selected to address relevant MDC mechanisms with the aim of combining them in an integrated approach to testing and assessment (IATA) specifically tailored to MDCs.

The results of interlaboratory prevalidation and augmentation studies of test methods modelling CYP enzyme induction, adipogenesis and peroxisome proliferator-activated receptor activation will be presented. Generally, the prevalidation activities successfully demonstrated that the methods were well standardized and ready for further validation. However, challenges related to relevance assessment and reproducibility across laboratories were observed. These will be highlighted and recommendations to resolve them presented.

GOLIATH will be pivotal in the development of an internationally harmonised strategy for testing MDCs and contribute to assessing chemical metabolic disruption hazards to help to reduce the worldwide rise in metabolic disorders that have reached "Goliathan" proportions.

This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement GOLIATH No. 825489.

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Evaluation of antiviral treatment and host immune response during parainfluenza 3 virus (hPIV-3) infection in human precision-cut lung slices

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Parainfluenza 3 virus induces severe infections in immunocompromised patients, which results in pneumonia and bronchiolitis. The infection is associated with virus transmission to the lower respiratory tract, and no antiviral treatments are available yet. To enable development of antiviral therapeutics, a better understanding and modelling of the pathogen-host interaction is essential. To replace animal experiments, we evaluated hPIV-3 infection in viable human precision-cut lung slices (PCLS) *ex vivo*.

PCLS were inoculated with 10^5 ffu/mL and post-incubated for up to 72 h post infection and prophylactically treated with a specific antiviral inhibitor. Virus localization within the tissue, viral replication, local inflammatory host immune response and gene expression profile were analyzed.

Ex vivo infected human PCLS by hPIV-3 showed a time-dependent increase in hPIV-3 virus titer in the supernatant, confirming active virus replication, which correlated with local immune response. hPIV-3 infection significantly induced secretion of key antiviral and pro-inflammatory cytokines such as IFN-α, IP-10, IFN-λ, IFN-γ, RANTES or IL-6 compared with uninfected control. Gene-expression profile revealed 193 differentially expressed genes (DEGs) mapped to innate and adaptive immune response upon infection. Novel antiviral treatment dose-dependently inhibited hPIV-3 virus replication and reduced number of DEGs showing promising results for future treatment.

We show for the first time that parainfluenza infection of human lower respiratory tract tissue can be mimicked *ex vivo* and thereby reduce number of *in vivo* experiments. This enables further in-depth evaluation of the T-cell specific immune response, which plays a crucial role in disease severity and can be used to profile antivirals.

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Refinement by gentle handling of mice affects pharmacokinetic end points

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There are limited studies which investigate the effects of laboratory animal handling and training (habituation) on scientific end points, e.g., pharmacokinetics, hindering implementation and refinement in both academia and industry. The purpose of this study was to investigate the effects of handling method (tail lifting vs tube lifting) and training (habituation to handling) on pharmacokinetic end points as well as welfare parameters in a preclinical pharmacokinetic study using a well-defined pharmaceutical. CD1 mice were either tail lifted without training, tube lifted without training or tube lifted with a 10-day training protocol. A pharmaceutical was then administered by oral gavage and a 24 h pharmacokinetics study was performed in an industry setting. Handling method and training affected the pharmacokinetic end points. The trained group had a higher maximum serum concentration (Cmax) and Cmax was reached faster compared to the other groups. The trained group also had 30% higher drug exposure, a critical measure of drug bioavailability, than tail and tube lifted groups. In addition, tube lifted mice had lower facial grimace scores than tail lifted mice, indicating lower levels of distress. Handler interaction after repeated blood sampling was highest in the trained group; only trained mice voluntarily touched and climbed on the handler after blood sampling. In conclusion, stress caused by tail lifting, oral gavage and blood sampling results in delayed drug absorption and reduced exposure. Stress could be reduced by gentle handling and habituation which may result in more relevant pharmacokinetic data, increased scientific quality and improved animal welfare.

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Evaluating skin irritancy of cannabidiol: A comparative study with reconstructed human epidermis models

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Reconstructed human epidermis (RHE) is one of *in vitro* skin irritation test methods. There are several validated RHE models in OECD test guideline 439 for the testing of chemicals. Most of them generate from normal human epidermal keratinocytes, however each model has a difference regarding age or race of donor. For instance, KeraSkin™ is constructed from Korean-originated keratinocytes. Therefore, it is necessary to investigate distinct differences between the models.

Cannabidiol (CBD) is a major compound derived from Cannabis Sativa, Hemp, and non-psychotropic plant unlike delta-9-tetrahydrocannabinol. CBD is known for anti-inflammatory, antioxidant, and anti-acne effects. It is possible to utilize medical cannabis under regulation free zone in Korea recently. We could obtain 99.6% CBD from hemp which was grown in smart farm for this study.

The purpose of the study is to evaluate skin irritancy of CBD with multiple RHE models, including EpiDerm™, SkinEthic™ RHE, LabCyte EPI-MODEL24 and KeraSkin™ according to the manufacturer's instruction. CBD isolate powder and cream were used as test materials. Tissue viability was measured by MTT assay. The medium from every model were collected and inflammatory cytokines (IL-1α, IL-6, TNF-α) were quantified by ELISA.

All test materials were classified as non-irritant (> 50%) in all RHE models. Notably, expression of TNF-α was about twice as high in KeraSkin™ compared to others. These results suggest that various RHE models show different results in cytokine secretion due to characteristics of donor or individual protocols unlike tissue viability.

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Tolerance of topical formulations on 3D reconstructed tissue models

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Local tolerance is a common pre-requisite before placing topical products on the market and its assessment is increasingly based on *in vitro* methods (EMA/CHMP/SWP/2145/2000 Rev.1). Validated methods are available to classify chemicals and mixtures, but they are not applicable to assess the irritation potential of complex mixtures, such as topical formulations to be applied on skin and epithelia.

ISO 10993-23:2021 has validated the use of reconstructed human epidermis to assess skin irritation potential of medical device (MD) extracts and components and the same approach (cytotoxicity assay) could be applied to assess epithelial irritation on other tissue barrier models if qualified in order to replace *in vivo* special irritation assays (e.g. corneal, oral, vaginal, rectal mucosae).

To support the relevance and predictivity of reconstructed tissues in topical formulations assessment, we analyzed the historical data available in our laboratory: different formulation types (emulsions, sprays, gels, surfactant-based formulations, ovules) intended to be used on oral or vaginal mucosa were considered. The experimental protocol was based on 1h exposure followed by product removal and 16 h recovery on Oral or Vaginal Human Epithelium.

SDS was correctly predicted with viability < 50%; formulations were classified as irritant or non-irritant to the oral mucosa or to the vaginal mucosa according to cell viability results alone or with other information gained on tissue integrity, morphology and inflammatory response.

These data confirm the relevance of the *in vitro* approach on 3D tissue models, its flexibility and applicability to formulations regardless their regulatory status.

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Monocyte activation test (MAT) as a tool to assess the indoor air quality (IAQ) in laboratory areas

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The evaluation of bioaerosols is very important for Public Health directly related to airway diseases and health effects. This study evaluated the applicability of MAT as a tool to assess the indoor air quality in laboratory areas at Fiocruz (Rio de Janeiro, Brazil). Air samples were collected in 3 laboratories and 2 offices and analyzed for fungi count (CFUm-3) and MAT/IL-1β, IL-6 (EEUmL-1). Results show that the outdoor fungal concentrations ranged from 673 to 1,530 CFU/m3 and the indoor count ranged from 212 to 825 CFUm-3. Fifty percent of samples showed CFUm-3 values above World Health Organization (WHO) and European Commission recommendations and 10% above recommended by the Brazilian Health Regulatory Agency (ANVISA). A range of 2.46 to 6.15 EEUmL-1 for IL-1β and 3.23 to 6.41 EUUmL-1 for IL- 6 were found when the outdoor pyrogenic activity was analyzed. The indoor pyrogenic activity results ranged from 0.16 to 5.17 EEUmL-1 for IL-1β and from 0.49 to 5.70 EEUmL-1 for IL-6. If we considered the value 0.5 UE/mL as positive control, 70% of the samples remained over this limit. Our data support that MAT is a rapid, reliable tool for measuring pyrogens that could be used as an indicator of IAQ. However, there is a need to establish limits for the total biological load (EEU) of the environment in order to know the possible health effects.

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Application of iPSC derived hepatocytes, cardiomyocytes, endothelial cells, renal podocytes and proximal tubular cells for predicting mitochondrial toxicity

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Predicting potential adverse effects of chemicals is a crucial component of the drug development process. Traditional animal-based testing entails issues related to human relevance and ethics. Moreover, with the rising evidence of the role of mitochondria dysfunction at the base of many organ toxicities, relevant systems capable to capture cellular and mitochondrial toxicities constitute an impelling necessity. Here, we evaluate the use of iPSC derived *in vitro* systems in combination with a battery of specific assays for the delineation of test substances' adverse profile in terms of mitochondrial toxicity.

iPSCs were differentiated in some of the most perfused organ cell types (kidney, liver, heart, and vascular system) and exposed to a set of ten compounds, including drugs known to induce organ toxicity, possibly via mitochondrial impairment. After 24 h of treatment, cytotoxicity (resazurin), oxygen consumption rates (OCR-Seahorse), glycolytic activity (lactate) and transcriptomics (TempoSeq) were assessed.

Decreases in OCR in the absence of cytotoxicity allowed for detection of mitochondrial liabilities of compounds. Comparison of OCR, cell viability and increases in lactate upon treatment with the same chemical, revealed different sensitivities amongst the various *in vitro* systems, possibly because of targeted toxicity. Moreover, analysis of gene expression via genome wide transcriptomics provided mechanistic insights on the substances' mode of action.

Successful detection of test compounds toxicity and their link to mitochondrial dysfunction, indicated that iPSCs derived models in combination with the proposed battery of *in vitro* assays may represent a useful tool to be implemented in the preclinical detection of chemical hazard.

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Human-induced pluripotent stem cell reporters for high-content screening of stress response activation identifying target organ-specific toxicities

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Development and validation of next-generation *in-vitro* test systems to improve predictions of chemical-induced adversity for specific target organs is critical. Human-induced pluripotent stem cells (hiPSCs) are a valuable source for studying chemical-induced toxicities in various organ specific cell types within the same genetic background. This enables the identification of organ-specific toxicities during chemical safety screening. Activation of stress response pathways is an early key event towards organ injury. Monitoring critical genes within these pathways would both give insight in the mode-of-action and aid in the prediction of liabilities for adverse outcomes upon exposure. Here, using the CRISPR/Cas9 technology we built a reporter panel in hiPSCs in which genes based on transcriptomic analysis for critical stress response pathways were endogenously tagged with eGFP. The hiPSC reporter panel covers inflammatory signalling, DNA damage, ER stress and oxidative stress response pathways. The activation of stress response pathways was monitored using live-cell confocal imaging following chemical exposure in multiple cell types after differentiation (e.g., hepatocyte-like cells, proximal tubular-like cells, cardiomyocytes). Differences in sensitivity towards chemical exposure between different organ-specific cell types could be observed. To study multi-cellular dependent responses and the utility of more advanced 3D systems, these reporters can be grown as organoids for both liver and kidney. This allows to study cell-specific responses within a multi-cellular environment more recapitulating the *in vivo* settings. We anticipate that stress response hiPSC reporters will help improve chemical safety testing allowing the identification of the mode-of-action as well as specific target organ or cell specific toxicities.

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Generation of a human 3D *in vitro* bone model that mimics glucocorticoid-induced osteoporosis

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Osteoporosis is a bone disease in which the bones become so weak and brittle that even a fall or a light strain, such as bending or stumbling, can lead to a fracture that affects the patient's mobility. Osteoporosis occurs when new bone formation by osteoblasts does not keep pace with the breakdown of old bone by osteoclasts. Several risk factors can increase the likelihood of developing osteoporosis – including age, race, lifestyle, and medical conditions and treatments such as glucocorticoids.

To simulate glucocorticoid-induced osteoporosis (GIOP) for *in vitro* drug testing, we aim to establish and characterize an *in vitro* "healthy" human bone model exposed to glucocorticoids and finally treated with drugs for osteoporosis.

Our model includes bone-anabolic osteoblasts and bone-catabolic osteoclasts seeded on β-tricalcium phosphate (β-TCP). The anabolic bone was generated by seeding mesenchymal stromal cells (MSCs) on β-TCP. To obtain seedable osteoclasts, we incubated peripheral blood-derived, CD14+-sorted monocytes using low-attachment plates for 21 days in ∝MEM, 5% human AB serum, 2 mmol L-glutamine, 25 ng/ml M-CSF, and 50 ng/ml RANKL. Differentiated cells were characterized in detail.

Multinuclearity, u-shaped ß-actin-ring formation, and osteoclast activity confirmed by positive TRAP-staining and positive resorption assay delineated successful functional osteoclast differentiation. These osteoclasts were seeded onto the MSC-seeded β-TCP construct, maintaining their functionality as confirmed by the secretion of RANKL, MMP9, and free phosphate.

Prospectively, we will transfer our "healthy" bone model by applying glucocorticoids into an osteoporosis (GIOP) model for preclinical drug testing to finally achieve an *in vitro* 3D drug testing platform.

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Staphylococcus aureus infection alters sterol metabolism and induces hypoxia in intestinal organoids

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Although *Staphylococcus aureus* is one of the most studied bacteria worldwide, little is known about its molecular pathogenesis during intestinal infections and its associated secondary functional gastrointestinal disorders.

The aim of this study was to investigate alterations in the sterol metabolism and oxygen content in induced pluripotent stem cell derived intestinal organoids and colonic intestinal stem cell-derived intestinal organoids, as well as Caco2 cells in response to *S. aureus* infections.

During infection there was remarkable reduction of cellular total cholesterol, quantified using HPLC. To determine whether this reduction in cholesterol is caused by intrinsic changes in the oxysterol pool, we analyzed 3 ring oxysterols and 2 side chain oxysterols. Distinct differences in oxysterol levels were confirmed upon infection. Next, we characterized intra- and extracellular oxygen level. Three different oxygen measuring approaches have been established to investigate the O_2 levels in the cells and organoids upon infection: (1) cell-penetrating nanoparticles to quantify intracellular oxygen level, (2) sensor plates to quantify the extracellular oxygen in the media and (3) sensor foil system for the distribution of the ambient oxygen in organoid cultures. All three methods revealed a significant intra- and extracellular drop of oxygen in both intestinal organoids upon infection. In summary, we show that intestinal infection with *S. aureus* leads to cellular hypoxia associated with changes in sterol metabolism, which might be the cause of altered protein trafficking. These findings will help to understand cellular stress responses during persistent bacterial infections in the intestinal epithelium.

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Improving the use of human tissue and cells within research and education to reduce animal experiments

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Research and education with human tissue and cells not only offers the possibility to substitute and complement animal experiments but can also improve the quality of research. The Dutch National Committee for the protection of animals used for scientific purposes (NCad) investigated the possibilities to improve the use of human material in scientific research by identifying barriers and opportunities and formulating advises for improvement. The study was based on an in-depth literature study combined with interviews of experts. We focused on a) national laws and ethical codes, b) findability, accessibility, and availability of human tissue and cells for scientific research, c) national initiatives working on improvements in this plane. We also compared the situation to other countries within and outside the EU.

We identified that many national laws regulate the use of human material for scientific and educational purposes, fragmenting the playing field and impeding the use of the material. The evaluation also highlighted that not only the availability of human material but also the findability of the material requires improvement. No large differences were observed with other EU and non-EU countries. Based on these observations we advise to: 1) better coordinate laws and regulation 2) streamline, improve and uniform the processes, infrastructure, and the access to human material 3) harmonize and improve exchange between bodies reviewing animal experiments and the use of human material. Implementing these advises can significantly contribute to the use of human material in education and research and contribute to the reduction of animal experiments.

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Simulating arthritis in an animal-free *in vitro* 3D synovial membrane model

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Osteoarthritis (OA) is the most common joint disease, with rheumatoid arthritis (RA) representing the most common autoimmune arthritis. Fibroblast-like synoviocytes (FLSs) of the synovium are critical drivers of arthritis. In contrast, macrophage-like synoviocytes (MLSs) have been implicated in maintaining barrier function. However, the impact of FLSs and their interaction with MLSs on cartilage degradation remains elusive.

Here, we aimed to develop an animal-free, human-centered, *in vitro* 3D synovial membrane model that reflects the (patho-)physiology of the synovium to study the course of arthritis and new therapeutic strategies.

Bone marrow-derived mesenchymal stromal cells (MSCs) were used to reflect the physiological state compared to FLSs from OA patients. Blood-derived monocytes were differentiated into either M1 or M2 macrophages, with M2 mimicking MLSs. Cells mimicking FLSs and MLSs were incorporated into a synthetic RGD hydrogel and stacked layer-by-layer, mimicking the lining and sublining layer in an animal-free *in vitro* 3D model.

Tumor necrosis factor-α treatment increased, e.g., FLSs proliferation reflecting pannus formation typical of RA. Transforming growth factor-beta and OA synovial fluid induced an OA-like fibrotic phenotype as evidenced by increased expression of ACTA2 (actin alpha-2, smooth muscle) and SERPINE1 (serpin family E member 1) compared to the untreated control. M2-MLSs in the lining layer significantly attenuated both effects compared to M1-MLSs.

Our *in vitro* 3D synovial membrane model allows for studying cytokine-driven cellular changes and cell-cell interaction in a defined manner. Prospectively, we aim to provide an *in vitro* alternative for preclinical drug screening by incorporating both M1 and M2 macrophages.

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Age and gender specific safety: Thyroid-mediated developmental neurotoxicity

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The Virtual Human Platform for Safety Assessment project (www.vhp4safety.nl, NWA-ORC 1292.19.272) aims to accelerate the transition from animal-based testing to innovative safety assessments by improving the prediction of harmful effects of chemicals and pharmaceuticals based on human biology. We present the VHP4Safety case study "Age and gender specific safety: thyroid-mediated developmental neurotoxicity". The aim is to develop an animal-free strategy to assess the safety of compounds for thyroid disruption-mediated developmental neurotoxicity. First, the existing physiological data on thyroid homeostasis and brain development is used to generate a mechanistic map for the thyroid-brain-axis. This map, together with existing epidemiological and clinical data, will be used to develop a (quantitative) adverse outcome pathway ((q)AOP) network. *In vitro* models with which critical key events in the (q)AOP can be assessed will be selected in order to design a test battery for thyroid-mediated brain effects. We developed an AOP network based on available data of existing thyroid related AOPs and human physiology. By combining this data, critical molecular initiating events and key events were identified and used as a basis for *in vitro* testing. Cell models that measure how chemicals affect the T3 and T4 levels in brain-specific cells such as neurons, astrocytes and oligodendrocytes have been developed. These models can be used to study the effect of compounds that disrupt the thyroid hormone balance in the brain. Taken together, the results will contribute to a testing strategy that can predict how chemicals affect brain development. Further research will include other *in vitro* models.

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Re-evaluating the need for chronic toxicity studies with therapeutic monoclonal antibodies, using a weight of evidence approach

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To support registration of monoclonal antibodies (mAbs) for chronic indications, 6-month toxicity studies have historically been conducted as per ICH S6(R1) guidance. Experience with mAb development has shown a relatively benign and well-understood safety profile for this class, with most toxicity findings anticipated based on pharmacology. Under an EPAA supported and funded project, a consortium of 14 pharmaceutical companies, the Medicines Evaluation Board (MEB) and the NC3Rs conducted a study to evaluate whether a 6-month toxicity study is still necessary to assess the long-term safety of mAbs. Data on First-in-Human (FIH)-enabling and chronic toxicity studies were shared for 142 mAbs submitted by 11 companies. Opportunities to further optimize study designs to reduce animal usage were identified. For 71% of mAbs, no toxicities or no new toxicities were noted in chronic studies compared to FIH-enabling study findings. New toxicities related to exaggerated pharmacology or ADA-mediated (not considered of human concern) were identified in 15.3% of cases. New toxicities of potential concern for human safety or that changed trial design were identified in 13.5% of cases, with 7% being considered critical and 2% leading to program termination. An iterative, weight-of-evidence model which considers factors that influence the overall risk for a mAb to cause toxicity was developed, to drive selection of the optimal duration of toxicity study without defaulting to a study of 6 months duration. This model enables an evidence-based justification, suggesting when 3-month toxicity studies are likely sufficient to support late-stage clinical development and registration for some mAbs.

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Tissue-specific network analysis to predict the hepatotoxicity of chemicals

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Development of new approach methodologies to characterize a potential hazard of cosmetic ingredients is a pivotal topic since the animal testing ban. Qualitative and quantitative methods to identify biological similarities, mechanism of action (MoA) and point of departure (PoD) are needed. Omics data have emerged as valuable source of knowledge to address these topics. In recent years, toxicogenomic datasets emerged to document systemic gene expression and their downstream effects on toxicological endpoints. Among the many options, network analysis appears to be very promising computational methods and have been proven useful for integration and exploration depicting complex interactions. In this work, we used network analysis to characterize and evaluate the hepatotoxicity level of chemicals.

We constructed a multi-layer, liver-specific molecular network by integrating multi-omics datasets including gene expression of human HepG2 cell line, protein-protein interactions, drug targets, pathways and adverse events. An analytical framework consisting of network signal diffusion and module detection was developed to help predict mechanisms of drug-induced liver toxicity. This approach was benchmarked on gene signature of hepatotoxic and non-hepatotoxic compounds obtained from transcriptomic dataset.

Liver specific networks analysis allowed to go beyond conventional transcriptomics exploration by 1) refining enrichment and highlighting relevant MoAs, 2) connecting genes and pathways to proteins, drugs and adverse effects to enrich biological interpretation and 3) detecting modules allowing quantification of pathways activation, paving the way for PoDs.

This framework has been packaged in an R tool that builds networks to identify potentially hepatotoxic chemicals and their MoA.

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The inter-individual toxicodynamic variability of cellular stress response transcriptomic perturbations upon chemical exposure

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Chemical safety assessment can be improved by accounting for inter-individual variability in toxicodynamic responses. An early key event of chemical-induced injury is adaptive stress response activation, a cellular mechanism to overcome stress. Given the diversity in adverse outcomes, mapping inter-individual differences within stress response activation is key. This could improve drug toxicity screening and help define data-driven safety factors accounting for variability. We profiled the transcriptome of a panel of 50 cryo-preserved primary human hepatocytes derived from different individuals exposed for 8 or 24 h to a broad concentration range of stress response inducers. Variances in concentration-dependent stress responses among individuals were captured, with average benchmark concentrations displaying maximum differences of 864, 13, 13, and 259-fold among different hepatocytes for UPR, oxidative stress, DNA damage and NF-κB signaling-related genes, respectively. Human population modeling revealed that small panel sizes systematically under-estimated variance and resulted in low probabilities in estimating correct variances among humans. Estimated toxicodynamic variability factors were up to 2-fold higher than standard uncertainty factors of 101/2 to account for population differences during risk assessment. Next, toxicodynamic transcriptomic variability among peripheral blood mononuclear cells from a large panel of healthy volunteers considering different age, sex and ethnicity, will be assessed following chemical exposures. Variability in weighted co-expressed gene networks will be combined with human population modelling to define data-driven safety factors for specific stress response networks. High-throughput transcriptome analysis combined with population modelling improves understanding of variability in stress response activation across the human population, thus aiding improved adversity predictions.

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Group versus individual housing in male and female laboratory mice: A multicenter study reveals only subtle differences in severity

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Finding ideal housing conditions for laboratory mice is a pivotal but challenging task. After all, mice are the most frequently studied animal model and their welfare must be promoted for ethical and scientific reasons. The required stable and harmonious groups housing as demanded by EU Directive 2010/63 very often turns out to be difficult. This poses a problem for the comparability between male and female mice, which can be kept more easily in groups. In most studies including both sexes, mice are housed in the same social condition, even though individual housing is presumed to be more stressful for female animals than for males.

We aimed to clarify the severity of group and single housing for each sex. Thus, this study serves to refine and optimize the quality of animal experiments.

We conducted a multicenter study at four sites (Aachen, Mannheim, Munich, Heidelberg) to provide an evidence-based severity assessment of group and individual housing in both sexes. The multicenter approach seeks improved robustness and external validity.

For stress assessment, we used animal behavioral and physiological parameters as indicators of severity. All data were recorded, centrally collected and statistically analyzed. We tested C57BL/6 mice from the identical litters simultaneously in all experimental sites with the same protocol. Despite the definition of controlled factors, standardized factors and standard operating

procedures between laboratories, the common observation that "mice fight more in some labs than in others" was confirmed. We found no or only subtle effects due to housing conditions, especially in female mice.

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How to assess the risk of chemicals causing skin sensitisation without using animals: The role that *in silico* tools play

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Cosmetic products require risk assessment for, amongst other adverse outcomes, potential skin sensitisation reactions. *In vivo* tests, such as the murine Local Lymph Node Assay and Guinea Pig Maximisation Test, have traditionally been used for risk assessment surrounding skin sensitisation. However, there is a substantial ethical and scientific impetus to move away from animal models when suitable New Approach Methodologies (NAMs) can adequately replace historical animal testing methods. Recent progress made in NAMs, coupled with advances within *in silico* skin sensitisation prediction capabilities, has enabled the development of non-animal Next Generation Risk Assessments (NGRAs). Herein, we describe a case study employing NAMs within an NGRA framework to provide a skin sensitisation assessment for benzyl benzoate using the following sources of information: a search of historical *in vivo* data (returning a mix of weakly sensitising and non-sensitising results), an *in silico* hazard prediction from Derek Nexus (returning an alert for benzyl esters), a Read Across prediction via a k-nearest neighbour model (predicting moderate sensitisation potential), an exposure-based waiving assessment using the Dermal Sensitisation Thresholds (identifying benzyl benzoate as reactive), and an integration of NAM data via Lhasa's ITSv1 Defined Approach Web Tool (returning inconclusive results, corroborating results from other Defined Approaches). When the available NAM data is considered in an overall Weight of Evidence, benzyl benzoate is classed as a GHS 1B sensitizer, indicating weak/moderate sensitisation potential. The approach described above demonstrates the effectiveness of non-animal NAMs, including *in silico* methodologies, in the assessment of skin sensitisation potential.

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Considering xenotransplantation and other more viable solutions to the organ shortage

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Organ transplantation is the standard of care for end-stage organ failure, but the demand for organs surpasses supply and leaves patients waiting – over 100,000 per year in the United States alone. Efforts to resolve the organ shortage through increasing organ donations, optimizing matching, and improving organ recovery and transport are promising but have not been able to close the treatment gap. Recent advances in gene editing and pig-to-nonhuman primate transplantation have led to renewed attention in pig-to-human xenotransplantation as a viable alternative to organ transplantation. However, significant immunological, ethical, and economic concerns restrict xenotransplantation as a viable alternative, including risks posed to organ recipients, zoonoses, animal use, financial costs for patients and insurance payers, and the opportunity cost of diverting finite public funds away from more viable strategies. Despite these serious issues, researchers, medical practitioners, and ethicists generally operate under the assumption that xenotransplantation should proceed and have forged ahead. A more careful consideration of xenotransplantation and other solutions to the organ shortage is therefore warranted. This presentation will discuss these considerations and provide recommendations for shifting research and resources toward more tenable strategies. Namely, the need for transplantation can be averted by preventing the end-stage chronic diseases that land patients on the organ waitlist; by pursuing more immediate strategies like optimizing living donation, improving the diagnosis and management of organ rejection, expanding donor criteria, and shifting to opt-out donation systems; and by investing in the development of safer, more ethical biotechnological solutions like organ bioprinting.

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Mechanisms of metal exposure on neuronal differentiation in the human neural progenitor test

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Current safety testing of compounds for developmental neurotoxicity (DNT) is performed in animal experiments, if performed at all, which has limitations and ethical issues. There is, therefore, a need for animal-free testing strategies that better mimic human physiology. Human-based stem cell assays are promising models, since they mimic cellular events that occur during early brain development, especially processes related to neuronal differentiation.

Here we investigated the ability of the human neural progenitor test (hNPT) to discern mechanisms of action of three metals that are known to affect brain development: methyl mercury (II) chloride (MeHg), lead acetate (PbAc) and sodium (meta) arsenite (NaAs). The hNPT consists of human stem-cell-derived neural progenitor cells that are differentiated to a neuron-astrocyte co-culture in ten days. During this differentiation, hNPT was exposed to the compounds at concentrations below effects on cell viability. Whole transcriptome analysis was performed using RNA-Seq. GO-term enrichment showed that MeHg affected gene expression relating to synaptic signalling, while PbAc did not regulate specific neuronal GO-terms. Signalling Pathway Impact Analysis on the data revealed that PbAc may affect axon guidance and confirmed the effects by MeHg on synaptic transmission. NaAs did not show enriched GO-terms or pathways at non-cytotoxic concentrations, suggesting that the mechanistic action of this compound may be outside the applicability domain of hNPT. These results show that hNPT may be a suitable model to detect specific DNT effects of compounds and further defines its biological domain. This contributes to determine the potential place of hNPT in testing strategies.

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Using *in vitro* data and PBPK models to predict inhalation toxicity

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In vitro assay data can be used to predict safe chemical exposure levels when combined with reverse dosimetry that utilizes pharmacokinetic data. We conducted a proof-of-concept study to evaluate using non-animal approaches to inform inhalation hazard identification and assessment. We selected 20 volatile organic compounds (e.g., styrene, tetrachloroethylene) for evaluation based on the abundance of pharmacokinetic data and availability of published minimal risk levels derived from inhalation exposure studies. We obtained from public databases activity concentrations that were derived from *in vitro* assays measuring diverse endpoints (e.g., genotoxicity, cytochrome p450 activation, transcriptome analysis). Using these data, reverse dosimetry was performed with open-source and commercial PBPK modeling tools to estimate daily equivalent administered doses (EADs) that would result in plasma and lung concentrations equivalent to the *in vitro* activity concentrations. The EADs were then compared to the *in vivo* point of departure (POD) or published minimal risk levels. Our preliminary results showed that the estimated EADs based on lung concentration were closer to *in vivo* PODs than those based on plasma concentration for most chemicals. The differences between EADs and *in vivo* data varied greatly among chemicals and across assays. The EADs estimated using *in vitro* assays measuring endpoints more mechanistically relevant to *in vivo* toxicity better predicted *in vivo* PODs than EADs estimated using *in vitro* assays measuring nonspecific effects (e.g., cytotoxicity assays). This project demonstrates a promising approach for predicting inhalation toxicity using non-animal approaches.

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Acute fish cell toxicity testing in real-time with the RAINBOWflow CHIP biosensor

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We developed a biosensor for real-time testing of acute toxicity of chemicals and water samples using fish cells under flow conditions. Permanent fish cell lines can accurately and reproducibly predict fish acute toxicity, as illustrated in ISO 21115 and OECD TG249 using the rainbow trout (Oncorhynchus mykiss) RTgill-W1 cell line. However, these tests are carried out under static exposure conditions and assess cell viability only at a single time point, after chemical exposure. Thus, no information is gained on how toxicity develops over time. For our RAINBOWFLOW CHIP, we use the RTgill-W1 and the RTgutGC epithelial cell lines from rainbow trout in a microfluidic setup. The fluid flow creates a shear stress, which is a physiological stimulus to epithelial cells. Cell viability is monitored continuously during exposure by Electric Cell-substrate Impedance Sensing (ECIS); this method measures the resistance created by cells adhering to small electrodes to an electric current. The resistance reflects their health status; it can be measured quickly and non-invasively. By testing a set of model compounds with different physicochemical properties and toxic modes of action we show the wide applicability of the system, with different chemicals showing different toxicity profiles over time. Moreover, the biosensor can be used for the testing of water samples, and an extension for on-line water quality monitoring in the field is also currently being implemented. In conclusion, the RAINBOWFLOW CHIP biosensor is a versatile setup for *in vitro* acute toxicity testing which includes a range of factors previously neglected.

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In silico NAMs for nanomaterials: Where have you been, where are you going to?

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In 2023, at the request of the European Chemical Agency (ECHA) and the European Union Observatory for Nanomaterials (EUON), we conducted a comprehensive review of NAMs available for nanomaterials. This included evaluating more than 140,000 research papers, 208 deliverables from 23 EU projects, and 140 other documents (test guidances, protocols, etc.). 44 of the 221 identified NAMs referred to computational (*in silico*) methods.

This talk aims to summarize state-of-the-art and discuss perspectives of further development of nano-specific *in silico* methods. We will present examples of computational models for predicting properties important for nanoparticle safety assessment, including models previously developed in our group. Special attention will be put on the need for an appropriate representation of the nanomaterials' structure and the influence of the system (environment) on these properties. Finally, we will deliberate on necessary needs and directions for updating the EU-US Nanoinformatics 2030 Roadmap.

Presentation: Poster

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Comparison of points of departure in development of an *in vitro* to *in vivo* developmental toxicant identification workflow

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New approach methodologies (NAMs) represent one of the most promising avenues for the reduction and/or replacement of animals in toxicology. While there have been significant strides in the generation and evaluation of NAMs in the past few years, there are still some notable gaps in their coverage, including in the determination of developmental toxicity (DevTox). Estimation of DevTox potential will require consideration of biokinetic and biodynam-

ic factors resulting from maternal changes and fetal development. We are currently developing a workflow for *in vitro* to *in vivo* extrapolation of DevTox effects. The aspect of the study described here quantitatively evaluated available DevTox endpoints by comparing the traditional points of departure (in mg/kg/day) using DevTox *in vivo* data (PODTrad,DevTox) and NAM-based PODs using all available *in vitro* data (PODNAM,all) or DevTox data (PODNAM,DevTox) from the CompTox Dashboard. Of 26 chemicals that have been well-studied for developmental toxicity, 21 had at least one *in vitro* assay identified as having a DevTox endpoint. When comparing assays with different levels of biological complexity, cell-based and whole organism assays had similar AC50s (50% active concentrations) while protein-based assays had higher AC50s on average. The PODNAM,DevTox was more conservative than the PODTrad,DevTox 67% of the time (10/15, where 15 chemicals had *in vivo* data identified as having a developmental endpoint). Including all bioactivity assays, PODNAM,All was conservative 87% of the time (13/15). We are currently working to optimize the categorization of DevTox assays to more accurately predict potency of potential developmental toxicity via different modes of action.

Presentation: Poster

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Modulation of T-cell immune response in precision-cut intestinal slices from IBD patients *ex vivo*

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Inflammatory bowel diseases (IBD) are chronic, gastrointestinal disorders with steadily increasing prevalence. The unmet medical need for treatment of IBD patients requires the development of human-based immunocompetent models that contribute to translational knowledge. Moreover, the use of models that accurately replicate the human disease state are essential and can reduce animal experiments. Using viable, ultrathin tissue sections (precision-cut intestinal slices; PCIS), which contain all relevant intestinal cells in their native microenvironment, we aimed to assess local T-cell reactivity in PCIS of IBD and non-IBD patients *ex vivo*.

Therefore, we treated PCIS, prepared from human ileum resections, *ex vivo* for 24 h with T-cell activating as well as anti-inflammatory agents (e.g., Pimecrolimus). Tissue viability was analysed by ATP-assay and cytokine release via ELISA. PCIS morphology was analysed using hematoxylin-eosin staining and immunofluorescence.

PCIS showed disease-relevant differences such as disrupted epithelial barrier and accumulation of immune cells, especially CD4+ and CD8+ T-cells in IBD-PCIS. Upon T-cell specific stimulation, IBD-PCIS released higher levels of T-cell cytokines such as IL-2 (~2.5-fold), IL-10 (~3-fold) and IL-17A (~7-fold), compared to non-IBD tissue. These effects were inhibited when PCIS were treated *ex vivo* with anti-inflammatory drugs demonstrating that the immune response can be modulated in our model.

In conclusion, our results confirm increased presence and activity of T-cells in IBD-PCIS and their immunological activity is also modulable. Human PCIS offer great potential for investigating disease-specific markers with high translational relevance *ex vivo* and to test pharmaceutical interventions without animal experiments.

Presentation: Poster

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Using masculinized brain organoids to study the male bias in autism spectrum disorder

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While the brains of men and women are largely very similar, some profound sex-specific differences arise during neurodevelopment, which, e.g., manifest in the occurrence of neurological disorders. In the case of autism spectrum disorder (ASD), a polyetiological neurodevelopmental disorder, males are four times more likely to be affected than females. Suspected to be the basis of those differences in humans are androgens, which induce the masculinization of the prenatal male brain, while the female brain is developing in the absence of androgens. In contrast, in rodents, estrogens are conveying the masculinization of the brain.

Since the underlying pathways are human-specific, we use human brain organoids to model the masculinization of the brain *in vitro*. For this, brain organoids were generated from human iPSCs and then exposed to sex hormones during the sensitive period of their differentiation. We analyzed the expression of selected recep-

tors and enzymes as well as targets of androgen signaling to further investigate the pathways of brain masculinization, which can be recapitulated effectively in human brain organoids. Furthermore, cellular features related to the sexual differentiation of the brain and perturbed in ASD such as synaptogenesis and excitatory/inhibitory balance are investigated in organoids derived from either typically developed donors or from donors that were diagnosed with ASD.

Thus, human brain organoids are a promising model to investigate the role of sex hormones in the development of autistic features and can contribute to understanding the male bias in ASD.

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Presentation: Poster

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ReplacEd – How we educate on the use of non-animal methods as an animal welfare organisation

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Background: Young scientists often find themselves at a crossroad, in deciding whether to perform animal experiments as part of their research, or whether to focus on non-animal methods (NAMs). As an animal welfare organisation we aim to inspire young scientist about the possibilities of NAMs by providing education (for the past 10 years). As an NGO we have the unique position to provide independent education on NAMs and the transition towards animal-free innovations.

Method: Each year we give around 35 lectures with a debate component on NAMs within the challenging environment of the Laboratory Animal Science Course at universities throughout the Netherlands. We measure the impact and effect of our education amongst the students of this course, through a long-term survey, which we started in 2015 and are continuing in 2023, to retrospectively compare results.

Results: Although students take part in this course to receive certification to perform animal experiments, we have seen great enthusiasm from students to discuss NAMs. Through the first iteration of the survey, we have already learned that around 70% of students changed their opinion after the lecture. And we have witnessed changes in behaviour of these students, with students changing the focus of their scientific career path and even students applying for funding with our organisation. Results of the 2023 survey will be presented.

Highlight statement: Education on NAMs inspires young scientists and gets them excited about NAMs and the transition towards animal-free innovations, even within the challenging environment of the Laboratory Animal Science Course.

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Five years of 3R research funding: Measuring the impact

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Bringing about a cultural change in biomedical research, which embodies the 3Rs principles, is part of the vision of Charité 3R, which is an infrastructure of Charité – Universitätsmedizin Berlin. A total budget of 6 Mio € for Charité-internal 3R research projects has served as an extraordinary measure striving for this vision.

The overall idea of this 3R funding program is to close existing funding gaps and provide start-up money to overcome the persistence of established methods. Importantly, funding intends to enable researchers to subsequently apply for external funding to implement 3R approaches. During the last 5 years, 15 calls tailored for different stakeholder groups were developed to account for different 3R-related needs. Calls for "Refinement" or "Adding 3R Value to ongoing funded research projects" were repeated, while others were unique, such as the "3R Collaborative Research Network" call or the "Tandem Projects" call combining two young scientists from different disciplines for improving robustness of a 3R method. Independent external evaluation of the proposals ensured high quality and competitive research projects, while each evaluation process was specifically designed towards the aims of the respective call.

The projects were funded with a significant budget – but how to measure its impact? Quantitative measures show quite a successful performance – e.g. 49 publications in peer reviewed journals until today. However, an additional qualitative evaluation that depicts the individual impact for single researchers and research fields is challenging. We go for it and look forward to a fruitful discussion on our approach.

Presentation: Poster

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The future of pyrogenicity testing: Phasing out the rabbit pyrogen test

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The European Pharmacopoeia is, after 50 years of animal-based testing for pyrogenicity, proposing to withdraw the rabbit pyrogen test (RPT) from its texts (including for vaccines and biologics, 59 texts) by 2026. An international conference jointly hosted by the European Directorate for the Quality of Medicines & HealthCare (EDQM), Council of Europe, and the European Partnership for Alternative Approaches to Animal Testing (EPAA), was held in February 2023 to present how pharmaceutical partners (manufacturers, regulators, testing laboratories) are managing the transition to *in vitro* replacements of the RPT, the monocyte activation test (MAT) and bacterial endotoxin test (BET) and the challenges encountered in the process. The conference emerged from good collaboration of regulators and industry in the EPAA "Harmonisation of 3Rs in Biologicals" team. It gave participants the opportunity to share their experience with the MAT during a training aiming to stimulate widespread interest in the transition and ultimately ensuring that this major replacement is brought to a successful – and global – conclusion. The sessions allowed to show that technically, *in vitro* methods such as the MAT and the BET can completely replace the RPT. Pharmacopoeias from other regions (Brazil, China, India, Japan, United States) also joined the event and committed to progressively phase out the RPT from their respective rules, even though they are not as advanced as the Ph. Eur. on this journey. Also the WHO represented at the conference confirmed a similar path – promoting MAT over RPT – will be followed on a worldwide level.

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An AOP for oxidative stress in plaque formation

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Adverse outcome pathways (AOPs) have been developed as a risk assessment tool for regulatory applications. These AOPs describe a logical mechanistic sequence of events, starting with a Molecular Initiating Event (MIE), and ultimately leading to a clinically adverse outcome via a series of Key Events (KE). The AOP framework provides a system to make predictions and assessments while reducing the need for *in vivo* assessment.

In the absence of epidemiological evidence, assessment of the health effects of a product, chemical or therapy on the progression of atherosclerosis traditionally has necessitated long term exposure studies on animals such as the Apolipoprotein E deficient mouse.

We followed Organisation for Economic Co-operation and Development (OECD) guidelines to formulate and propose an AOP for atherosclerotic plaque progression, collating the evidence characterizing how cigarette smoke-induced oxidative stress can lead to a MIE. We describe a downstream pathway that includes multiple KEs such as the upregulation of proinflammatory mediators, nitric oxide depletion and endothelial dysfunction. Alterations indicated by these KEs can lead to plaque formation, progression of cardiovascular disease and increased risk of morbidity and mortality.

Identifying preclinical processes and clinical biomarkers associated with these KEs provides a framework for *in vitro* and clinical data analysis, supporting our AOP as a tool for regulatory assessment. The AOP provides a powerful alternative to animal experimentation for the assessment of atherosclerosis initiation and progression risk.

Presentation: Poster

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NGRAroute: A PARC roadmap for implementing next generation risk assessment (NGRA) in EU chemicals legislation

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Although there has been significant progress in the development of "New Approach Methodologies" (NAMs), almost all major chemical legislations in Europe rely substantially on animal testing. One of the goals of the Partnership for the Assessment of Risks from Chemicals (PARC) is to promote "Next Generation Risk Assessment" (NGRA), to replace *in vivo* testing in the future. Within PARC, we develop NGRAroute, a roadmap to implement NGRA as the default risk assessment approach in various EU chemical legislations.

Here we share a first overview of the next steps of the roadmap development. The basic preparatory work for this roadmap has been completed, including a review of existing work on NGRA and its contextual domains. Relevant EU chemical legislations were mapped with respect to risk assessment workflows, associated risk assessment questions, and an analysis of how these could be transformed to NGRA. Moreover, a collaboration has been established with the Horizon 2020 cluster ASPIS (Animal-free Safety assessment of chemicals: Project cluster for Implementation of novel Strategies, https://aspis-cluster.eu/). ASPIS is currently developing a generalised NGRA framework, which will provide the structural basis for the further roadmap work and, ultimately, the legislative uptake of NGRA.

In the next phase, "science-to-policy dialogue" (S2PD) networks will be built to directly involve European and global stakeholders from the research, regulatory and policy domains, who have a key role in applying and changing the existing chemical regulations. This will ensure that the roadmap represents a broad consensus within the chemical risk assessment community.

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Animal-free *in vitro* assessment of endocrine effects including phase-1 metabolism

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For the evaluation of mechanism-specific effects *in vitro*, animal-derived reagents like fetal bovine serum (FBS) and rat liver homogenate (S9) are frequently used. However, these components can affect bioassay quality due to inherent product variability.

The aim of this study was to perform two *in vitro* OECD guidelines for the detection of estrogen (OECD 455) or androgen (OECD 458) receptor agonists and antagonists without using animal-derived reagents. For this, the respective cell-lines ERα-CALUX® and AR-CALUX® were cultivated in chemically defined medium (CDM) without FBS. Since no simulation of *in vivo* biotransformation processes is included in either OECD guideline but can be important in the context of endocrine disruptors, a biotechnological S9 homogenate was implemented in both methods and validated with model compounds showing an altered endocrine activity after biotransformation.

Both cell lines could be adapted to a commercial CDM and responded to the respective reference compound (17β-estradiol and dihydrotestosterone). Additionally, the inclusion of S9-homogenates was evaluated in the agonistic ERα-CALUX® assay and bioactivation was observed for the model substance Benzo[a]Pyrene. However, the cells showed an increased doubling time and a reduced sensitivity to the reference compounds after adaptation to CDM. Modifications such as an optimized CDM composition and an improved test design will be evaluated. Additionally, more model substances will be tested in combination with the biotechnological S9 homogenate according to OECD 455 and 458.

Overall, the animal-free conduction and consideration of metabolic processes provide promising advantages for a more reproducible endocrine activity testing closer to the *in vivo* conditions.

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Exploring the predictability of tiered alternative tests in fish acute toxicity

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For the acute toxicity of fish in environmental toxicology tests, a single surrogate test cannot completely replace animal tests. Therefore, a large number of animals are used to evaluate the toxicity of substances every years. Currently, a layered strategy is adopted to estimate the toxicity of substances *in vitro* to *in vivo*, an efficient prediction method that avoids the use of animal tests. Therefore, the purpose of this study is to establish a Sequential Testing Strategy (STS) for evaluating acute toxicity in fish and explore the predictive power of STS. In here, we established STS for acute fish toxicity and further estimate the toxicity of the substances. The hazard identification accuracy rate of using the literature to verify that the substances were applied to STS was 84.62%, of which 61.54% of the substances used the data of the substitution test. The STS accuracy rate was 70.59%, and 61.54% of the substances were not used to estimate *in vivo* toxicity. In current study, a STS for acute toxicity testing of fish was established through the concept of stratified testing strategy to evaluate the toxicity of substances. We verified that the hazard identification accuracy rate of STS was as high as 84.62%, and the application of STS greatly reduces the number of animals used. In summary, this study provides a STS for acute toxicity tests in fish, which will contribute to further improving the predictive power of single surrogate tests for the development of ecotoxicology.

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Evaluation of running wheel behavior as a reliable marker for severity assessment and humane endpoint detection in a rat model with intracranial tumor

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In rodent models with intracranial tumor formation severity assessment and humane endpoint determination are fundamental for ethical and legal reasons. We here evaluated the suitability of running wheel behavior to classify the severity after surgery for tumor cell injection and resection, as well as for humane endpoint detection after tumor regrowth compared to body weight and clinical state.

Male BDIX rats (n = 11) were single-housed in cages equipped with a running wheel. Under general anesthesia, glioblastoma BT4Ca cells were stereotaxically injected into the frontal cortex. After eight days, the tumor was microsurgically resected. Body weight and running wheel behavior were monitored daily until humane endpoint criteria of sudden weight loss and deteriorated clinical state were reached.

On average, body weight and running wheel behavior, but not clinical state, were significantly reduced after surgery for cell injection and for tumor resection ($p < 0.05$). However, on the day of the endpoint, a sudden weight loss was accompanied by a deteriorated clinical state and reduced wheel running compared to the previous day ($p < 0.05$). Furthermore, weight loss at humane endpoint was more severe than after cell injection or tumor resection ($p < 0.05$). In contrast, reduced wheel running behavior at the endpoint did not differ from that after cell injection and tumor resection.

Together, the monitoring of wheel running behavior enabled severity classification in a rat model with intracranial tumor formation but was not superior in endpoint detection to body weight determination.

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Presentation: Poster

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Training and implementation of a reconstructed human epidermis (RHE) model to evaluate skin irritation in Argentina

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The implementation of alternative methods to the use of animals for regulatory purposes in Argentina is incipient. Although there are still no restrictions at the regulatory level, social pressure and advances in regulatory matters in other countries have led to the need for non-animal methodologies for the evaluation of cosmetic products. Faced with the global trend of replacement, the first Alternative Methods Laboratory (LMA-EBAL) was created in Argentina with the purpose of implementing, developing and training *in vitro* methodologies. In this sense, we organized the first hands-on workshop of SkinEthic™ OECD TG 439 for Skin Irritation Test in the country. The training was attended by regulators and specialists from the National Administration of Medicines, Food and Medical Technology (ANMAT), from the ANLIS Malbrán Institute, CONICET researchers, professors from the UBA, from the pharmaceutical and cosmetic industry, test laboratories and representatives of the Argentine Chamber of the Cosmetic and Perfumery Industry (CAPA). In the first implementation of the methodology in the LMA-EBAL, the skin irritation potential produced by 14 AIN VEGAN cosmetic products was assessed according to the OECD TG439 guideline. The success of this experience is important for Argentina since the training was very popular and the implementation allowed testing the SkinEthic import system from Episkin Brazil.

Presentation: Poster

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R2N – Animal replacement for basic research in the field of infectious diseases and inflammation

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While in many countries research animal numbers are declining or stable over the last years, the proportion of those used for basic biomedical research is increasing. Basic biomedical research has its own peculiarities: it is hypothesis driven, has often an explorative character, and research methods are highly flexible compared to classical safety or toxicology testing. *In vitro* methods are mainly used for addressing specific research questions rather than to replace animal experimentation. To overcome this, approachable and complex *in vitro* models are necessary allowing scientists to tackle their specific research objectives with complexity. Here, we report of a consortium consisting of 15 groups in Lower Saxony, Germany, that has established an academic network to develop test-systems for a better understanding of molecular pathomechanisms, cellular interactions and pathogen-host interactions during infection and inflammation. The focus lies on stem cell derived models for the digestive and respiratory tract. The *in vitro* models have been applied in SARS-CoV2 as well as other viral and bacterial infection research. We present our approaches, show outreach activities and would like to stimulate others to follow the path of animal free technologies utilized in basic academic research.

Presentation: Poster

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All for one: The research consortium FOR2591 aims at evidence-based assessment of wellbeing – A path towards effective refinement in animal-based research

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Enhancing good wellbeing of laboratory animals and limiting negative outcomes of experiments are the fundamental steps in refinement approaches. However, how can we measure wellbeing of and the impact of procedures on animals? To tackle this question, the German Research Foundation-funded research unit "Severity assessment in animal-based research" (https://severity-assessment.de/) was established, which is currently in its second funding phase. This multidisciplinary consortium focuses on evidence-based parameters to determine the wellbeing-status of animals, especially in gastro-intestinal, psychiatric, neurological, and surgical models. The consortium has systematically evaluated 65 parameters in 55 models, developed various data science-based approaches to enable objective severity grading, analysed refinement strategies including appropriate analgesia, and developed and integrated new technologies for home-cage-based welfare assessment – resulting in more than 120 publications so far. We present the research unit to underline the strengths of such a consortium to elucidate the complex task of evidence-based welfare assessment and stimulate others to contribute to this field.

Presentation: Poster

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Management of hand bites in nonhuman primate studies

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Nonhuman primates (NHPs) play an important role in scientific development, and with the recent COVID 19 pandemic, the number of researchers using NHPs has increased. However, accidents are always possible during NHPs studies, most common of which are bites. This study describes the procedures in managing both the personnel and animal in a bite to the hand situation. An incident happened while the personnel was manually restraining the *Macaca fascicularis*. Immediately after the bite, the wound was washed with sterile saline and the personnel was transported to the hospital. After testing the sensitivity of the wound to antibiotics, the damaged area was sutured and the personnel recovered after a three-week period. The animal had its canine teeth amputated to ensure personnel safety and to reduce its aggression. In addition, blood samples were collected from both the personnel and the animal to confirm herpes B virus infection, and the test results were negative. The animal was observed for over six months after the incident, and no abnormalities were found. Twelve months have passed since the accident, the victim has recovered well and is working without any after-effects. Experiments using NHPs always carry the possibility of accidents, and in some cases, they can be a serious problem. Therefore, each facility must have standard operating procedures for emergency treatment in case of accidents, and plans for the treatment of personnel and animals. We hope that this case can be helpful to institutions conducting research using NHPs in the future.

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Neurotoxicity effects of bifenthrin by zebrafish embryo model

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Bifenthrin is a pyrethroid insecticide used to treat a variety of crops. Nevertheless, studies on the bifenthrin-induced toxicity on aquatic organisms are still insufficient. In this study, the neurotoxicity of bifenthrin was investigated using the zebrafish embryo model, one of the possible alternative neurotoxicity test models.

In the present study, wildtype (WT) and transgenic (TG) [Tg(elavl3:EGFP) and Tg(mbp:mGFP)] zebrafish were exposed to 0, 0.1, 1, 11.1, 33.3, 100 and 300 µg/L for 120 hpf. Phenotypic endpoints including hatching rates, survival, morphology, body length, and weights were checked from 24 hpf. Behavior parameters, i.e., tail coiling, touch-evoked responses, and locomotor activity were also monitored. Furthermore, at 120 hpf, fluorescence imaging was done for the TG lines. Genes related to neurotransmitters and neurodevelopment were screened.

After 72 hpf, the 100 and 300 µg/L exposure groups started showing signs of abnormal morphology and body lengths. Moving duration, distance moved, body contact, and proximity were all significantly affected by bifenthrin exposure. Also, six genes related to neurotransmitters and eight related to neurodevelopment were significantly affected. Neurogenesis and myelination processes were disrupted as seen through fluorescence imaging with the TG lines.

From the results obtained so far, we found that bifenthrin can cause neurotoxic effects in teleost zebrafish and zebrafish establishes itself as an excellent alternative model for neurotoxicity testing.

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Developmental neurotoxicity *in vitro* assays applied for molecular initiation and key event identification to create an AOP network related to cognitive function defects

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Cognitive functions such as learning, memory, decision-making, problem-solving, and attention are key factors contributing to our personalities. They are established during brain development via a series of complex and interdependent processes that are particularly vulnerable to chemical insults. Despite its significance, only a fraction of our chemical exposome has been assessed for its developmental neurotoxicity (DNT) potential. For data gap closure by fast and cost-effective compound evaluation, a DNT *in vitro* battery (IVB) has been set up. To build confidence in IVB tests, the aim of this study was to map key neurodevelopmental events measured in neurospheres in combination with transcriptome analyses to putative adverse outcome pathways (AOPs).

Human 3D neurospheres were utilized to measure the effects of different compound classes on key neurodevelopmental events, i.e., neural progenitor cell (NPC) proliferation, migration, and lineage differentiation as well as thyroid hormone (TH)-dependent oligodendrocyte maturation by high content image analyses. These endophenotypic outcomes were combined with respective transcriptome analyses.

We identified three independent modes-of-action (MoAs) interfering with oligodendrocyte development (TH disruption via receptor binding, oxidative stress, and disturbance of cholesterol homeostasis) in differentiating NPC. Furthermore, we confirmed a migration endophenotype caused by compound-protein interaction. All identified KEs were integrated into already existing DNT-AOPs resulting in an AOP network related to cognitive function defects.

This study demonstrates the power of combining endophenotypic with transcriptomic analyses to elucidate compounds' MoA and create AOPs and AOP networks for gaining confidence in DNT hazard assessment by using the DNT IVB in a regulatory context.

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Assessment of human neural network formation and function using 2D and 3D hiPSC-derived cell systems

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Animal experiments are the current standard for developmental (DNT) and adult neurotoxicity (ANT) assessment of chemicals. As an alternative for regulatory DNT testing, an *in vitro* battery (DNT IVB) has been set up. One of the identified DNT IVB gaps concerns an assay for human neural network formation (hNNF) as this endpoint is currently only covered by rat cells. For ANT, there is no IVB available.

To close the DNT and ANT gaps, we developed three different human-induced pluripotent stem cell (hiPSC)-based assays to assess compound effects for DNT and ANT using microelectrode arrays (MEAs).

The hNNF assay forms functional neural networks from hiPSC-derived excitatory and inhibitory neurons and primary astroglia (Neucyte, USA) and challenging with 27 pesticides confirmed the suitability of the assay for DNT compound screening. Lately, also the hiPSC-derived mixed neuron-astrocyte culture-based human synaptogenesis assay established by the EU-JRC, was optimized for MEA activity using different media, coatings and cell numbers. For ANT, the human multi-neurotransmitter receptor (hMNR) assay based on hiPSC-derived 3D-BrainSpheres was set up. This test system is composed of different neuronal subtypes that can be quantified by sorting the detected spikes after acute compound treatment. These three test systems are currently refined by testing positive and negative compounds to determine their applicability domains and to improve their use for regulatory application.

In vitro assays assessing the formation and function of neural networks are useful tools for identifying compounds that might impair developmental or adult human brain function.

Presentation: Poster

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Web-based application for gathering knowledge about nano-specific NAMs in human safety assessment

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Over the last few decades, there has been a growing interest in the applications of engineered nanomaterials (ENMs) in a variety of consumer products. This forced the urgent need for the development of comprehensive and functional nano-specific approaches that would allow to assess human hazard of ENMs promptly. Considering the drawbacks of applying animal testing for this purpose in terms of its ethical, economic, and time limitations, delivering new approaches methodologies (NAMs) is of high priority. Enrichment and development of nano-specific NAMs was the objective of many international initiatives. In effect, many NAM-based strategies were developed.

Here, the goal is to present the newly developed, stand-alone, web-based application that collects available NAMs applied to characterize the hazard of nanomaterials. The application allows to search regulatory relevant nano-specific NAMs of interest in a timely manner and by using a different set of filters (e.g., NAMs relevant for the specific organ, NAMs crucial for specific EU regulation). It also provides detailed information about each NAM.

Such application can be helpful to the industry, academia, and other stakeholders who are interested in using nano-specific NAMs of regulatory relevance in human safety assessment.

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Skin sensitization reactivity data in a weight of evidence risk assessment for botanicals

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The marketplace has seen a consumer preference for products which contain botanical and other natural substances (BNS), as there is a perception that "natural" is safe. As with any consumer product ingredient, a thorough safety assessment must be conduct-

ed for all endpoints, including a determination of skin sensitization potential. However, challenges remain for BNS with variable composition, as available sensitization non animal test methods (NAM) are not suitable to assess complex mixtures and sensitization potency benchmark selection is often difficult.

To address some of the challenges, a modified Peroxidase Peptide Reactivity Assay (B-PPRA) was developed and recently applied in a proof-of-concept study for screening BNS for their ability to react with a synthetic peptide containing cysteine, as a surrogate for the first key event in the sensitization adverse outcome pathway.

In the study here, we explored how reactivity data, in particular the B-PPRA, could become part of a skin sensitization New Generation Risk Assessment framework for BNS. A weight of evidence (WoE) approach to evaluate BNS skin sensitization potential was applied integrating all available NAM information together with data on human topical use history, clinical and historical *in vivo* data as well as compositional information.

Several botanicals (e.g. Poison Ivy, Cashew, Chamomile, Milk thistle, Rosemary, Aloe Vera etc.) which cover a wide range of skin sensitization potential were selected as case studies and demonstrated how a WoE approach may increase data concordance and accuracy of skin sensitization prediction of BNS.

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Rapid and reproducible generation of novel cell lines

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A major limitation of *in vitro* studies is the shortage of high-quality cell models that are available in large enough quantities to allow large-scale experiments and cross-validation experiments. Immortalised cell lines may be an attractive option; however, traditional immortalisation technologies are unpredictable, cumbersome and can lead to drastic alterations in cellular physiology.

We established a novel immortalisation approach that relies on our CI-SCREEN library of immortalisation genes and enables the rapid generation of novel cell lines from almost any source material within 2-3 months.

We created more than 25 different cell types from more than 12 different species including Lung Epithelial Cells, Endothelial Cells, Astrocytes and Thyroid Epithelial Cells. We then characterised the novel cell lines and identified optimal immortalisation gene combinations for each cell type.

The novel cell lines showed unlimited expansion potential and were amenable to further genetic engineering. Immortalised cells maintained a primary-like phenotype and cell type-specific functions, which could be improved by 3D culture conditions (e.g., matrix-free spheroids, Air-Liquid-Interface) Once we identified optimal immortalisation gene combinations for each cell type, these combinations could be used to reproducibly immortalise the same cell type from different donors and samples.

This approach opens up the possibility to easily create personalised cell lines for disease-in-dish approaches. As the technology is species-independent, animal cell lines can be also easily created, allowing for large-scale, cross-species *in vitro* validation studies without the need to repeatedly take biopsies or samples from animals to obtain primary cells.

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Development and validation of an *in vitro* assay to assess extra mild personal care formulations and the generation of an industry-relevant benchmarking database

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Historically, *in vitro* tests for skin irritation were developed for analysis of harsh chemicals and don't have the sensitivity required to resolve differences in irritation potential between ultra-mild ingredients. Furthermore, validation of these historical tests used *in vivo* animal data, which can be over-predictive of human irritation, when compared to human patch tests.

Therefore, we developed a new test, adapted from a skin irritation ET50 test, which allows skincare formulations to be dosed mimicking real-life application. This adapted test enabled quantification of subtle differences between mild and ultra-mild formulations, that would not have been distinguished in the original method. These products can then be ranked by order of mildness.

We demonstrate the predictive capacity of the *in vitro* XtraMild test by directly validating it with human patch test data. In a series of blind-coded tests, the XtraMild *in vitro* assay accurately predicted the rank order of different surfactants and mild skincare formulations, to the rank orders obtained from the human patch test clinical scores.

We have generated a bench-marking database validated against human data, which provides valuable information to the industry, and allows results to be interpreted in the context of industry ranges for a specific product type.

This test is an effective pre-screen for formulations and ingredients relevant to the personal care industry. By using this test in the initial stages of product development, it can identify the mildest ingredient combinations, reducing the number of patch test volunteers required and their risk of developing an adverse irritation reaction.

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Acutox: An animal product-free assay for predicting acute oral toxicity

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Acute oral toxicity is currently assessed using the rodent LD50 test following OECD test guidelines 420, 423 and 425. These tests are widely criticized on scientific and ethical grounds, as they lack reproducibility and relevance to human exposure. We have shown that an *in vitro* cytotoxicity assay can be used to predict acute oral toxicity. The aim of this work was to develop an enhanced, animal product-free, metabolically relevant *in vitro* assay capable of predicting EPA and GHS acute oral toxicity category classifications.

Adult human dermal fibroblasts were grown in animal-product free media containing human serum and dosed with a cohort of 70 chemically diverse test articles with known, well curated rodent LD50 categories spanning all EPA and GHS classifications. Viability was assessed using both the Neutral Red Uptake and MTT assay, and IC50 calculated for all 70 test articles. These data were used to create a prediction model capable of predicting EPA classification.

This scientifically and ethically robust screening or replacement alternative to the rodent LD50 assay can be run alongside *in silico* approaches to improve the prediction of acute oral toxicity classification and labelling and is considered to be suitable for inclusion in a weight of evidence approach for acute oral toxicity classification. The Acutox assay contributes to a registrants 3Rs initiative by replacing aspects of *in vivo* acute oral toxicity testing and offers a novel animal product-free and metabolically relevant, human *in vitro* approach to acute toxicity.

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3R-SMART: An education and information platform to help reduce animal testing

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The long-term goal within the EU is to abolish animal testing (e.g., Recital 10 Directive 2010/63/EU) and – until this goal can be achieved – to strengthen the adoption of the 3R principles (replacement – reduction – refinement) in animal research.

While the EU aims to advance the 3Rs in the field of animal experimentation, training about alternatives to animal use only plays a secondary role in the education and training of people working with laboratory animals. Laboratory animal science (LAS) courses usually focus on reduction and refinement aspects to ensure that all staff working with laboratory animals is compassionate and competent.

"3R-SMART" (https://3r-smart.de) aims to bridge this gap by showcasing methodical approaches to reduce animal tests and by disseminating 3R research activities. The open access platform highlights specific efforts to replace, reduce, or refine animal experiments by providing instructive texts, explanatory videos, or recordings of lectures, 3R news and upcoming events as well as a 3R forum (registered users). Interactive maps introduce 3R centers in Germany and Europe.

In collaboration with LAS interactive (https://las-interactive.de) a common training portal about LAS and alternatives methods will be developed. In line with the idea to disseminate and advance knowledge about the 3Rs we are working on developing a 3R curriculum that can be integrated in laboratory animal science courses to support the development of 3R-competencies.

This way 3R-SMART forms an interface between animal research and alternative methods and can provide significant and sustainable support for a wider implementation of the 3R.

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Risk-based, geospatially informed prioritization of potentially cardiotoxic chemicals

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The cardiovascular (CV) system is significantly affected by environmental factors, but risk assessment of environmental chemicals is restricted due to limited data availability. New approach methodologies (NAMs) including *in vitro* and *in silico* methods for estimation of exposure and toxic effects, offer comprehensive approaches to replace animal testing. Here, we adopted physiologically based pharmacokinetic (PBPK) models-based workflow to convert activity concentrations of Tox21/ToxCast high throughput screening (HTS) assays relevant to six CV failure modes to human daily equivalent administered doses (EADs). To evaluate the human relevance of the predictions, these EADs were compared with exposure estimates derived from the USEPA ExpoCast program and *in vivo* animal study-based points of departure from ToxVal database. Geospatial mapping was used to assesses the potential risk of exposure for different populations across different counties/regions within the US. A group of potential cardiotoxic chemicals predicted to have low margins of exposure were identified. Chemicals such as Dibutyl phthalate, Perfluorooctanoic Acid and BisphenolA were identified with overlapping EAD ranges and exposure estimates, particularly within certain geographical hotspots. Incorporating geospatial distribution of chemicals allows for connection between molecular perturbations and adverse health outcomes across a population, enabling hypothesis generation and prioritization for further testing. Use of HTS assay outcome data in establishing a margin of exposure and POD ratio can advance the steps of human health safety evaluation by providing rapid, computationally supported screening-level assessments. This case study represents an advancement in the application of NAM based alternative approaches in human health risk-assessment for potentially cardiotoxic chemicals.

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Funding as a strategy: The role of private funding in growing the community around non-animal methods

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The Alternatives Research & Development Foundation (ARDF) Annual Open grant program was established in 1993 to fund research developing innovative methods to replace or reduce the use of animals in research, testing, and education. One of the longest-running programs of its kind, ARDF's Annual Open grant program funds investigator-initiated research across a broad – and evolving – range of scientific areas with a focus on projects with high scientific merit and potential to contribute to the replacement of animal models. This portfolio analysis examines the scientific areas and methods supported and the major scientific trends reflected in funding over the past ten years. We propose that private funding of even relatively small awards plays an important role in advancing non-animal methods.

Current awards are $40,000 for a year-long project, and the program invites proposals from non-profit educational or research institutions worldwide. The program has awarded over $4 million in funding and had an average funding rate of 21.5% for 2015-2022. Applications are evaluated by external reviewers with relevant expertise and ARDF relies on a diverse and dedicated cohort of reviewers from industry, academia, and government agencies who provide expert assessments based on the program's review criteria and scoring rubric. Continued progress in the development of non-animal research methods will require increased support and collaboration across the full range of funders: non-profit, commercial, and federal. This analysis examines one type of program and its role in supporting researchers who are working to reduce the use of animals in research and advance human health.

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Waste not, want not

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Research using genetically altered (GA) animals is responsible for the majority of animals which are bred in research establishments but then not used in research projects. Moreover, generating an ever-expanding number of new models contributes significantly to the total numbers of animals used in research. Dynamic breeding strategies, efficient cryopreservation or bio banking and effective resource-sharing can greatly reduce animal numbers. Progress in husbandry practices and the expansion of shared resources promises to deliver on this front. However, this potential is not being fully exploited due to challenges in overcoming established practices, issues around intellectual property and the lack of dissemination of resources.

As long as the use of GA animals is still considered necessary for some fields of research, those of us working in this field must focus on using the least number of animals for largest output of relevant scientific data. I will present the current challenges in doing so from the point of strategic planning to publishing and sharing resources. This will include those easier to overcome, such as the breeding strategies tailored to individual models and effective cryopreservation plans, but I will also discuss those more difficult issues. Sharing tissue, data or models themselves is often hampered a lack of technical ability, intellectual property rights and licences required to use new technologies. By discussing these issues, I hope to highlight ways to reduce animal numbers both directly by better husbandry practices but also indirectly by overcoming the hurdles to greater sharing.

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Cytotoxic and transcriptomic effects of pesticides on RTgill-W1 cells as an alternative approach for acute toxicity testing

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Ethical concerns and increasing demands for chemical testing has called for the need to explore New Approach Methods (NAMs) to reduce animal testing and address resource limitation issues. The rainbow trout gill cell line (RTgill-W1) was recently standardized by the Organization for Economic Co-operation and Development (OECD) to assess the cytotoxicity of chemicals. However, the test's focus on cytotoxicity does not provide much information on a chemical's mechanism of action. The first objective of this study was to use OECD Test No. 249 to characterize the cytotoxicity of 19 pesticides considered important to Canadian ecosystems. The second objective was to derive transcriptomic points of departure (tPODs) values for these same pesticides to gain a deeper understanding of the impacts these pesticides have on biological mechanisms. Cytotoxicity results from fluorescent dyes assessing cell viability found chlorothalonil, carbaryl, and diquat as the most toxic with LC50s of 21.2, 97.0, and 100.4 µM, respectively. Of the 19 chemicals tested, ten of them obtained LC50s below 500 µM. Studies are underway to derive tPOD values. This research demonstrates the potential for alternatives to animal testing strategies that are faster, more cost-effective, and yield more information.

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Towards establishing a microplate-based, transcriptomics assay for rainbow trout hatchlings

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There is interest in the development of early-life stage (ELS) tests with fish models that are high-throughput and can generate transcriptomics data. The objective of this study was to establish a method in rainbow trout hatchlings that could satisfy both of these interests. We based our pilot method on recent efforts by U.S. EPA researchers to establish a larval fathead minnow high throughput transcriptomics assay. Here, 1-2-day post hatch trout were assayed in 24 well microplates. Hatchlings were exposed for 24 hours to 11 different concentrations of three test chemicals (3,4-dichloroaniline – 0.003 to 10 mg/L; $CuSO_4$ – 0.15 to 5000 ug/L; ethinylestradiol – 0.03 to 100 ng/L) as well as negative controls (DMSO, culture water), with n = 12 per test concentration. Test concentrations were spaced on a half-log10 basis, with upper concentrations derived from a chemical's LC50 from the US EPA EcoTox database. Mortality was 2.2% during the transport and hatching phase. There was no mortality in the studies concerning dichloroaniline and ethinylestradiol, and none in the negative control groups. Based on the mortality observed from the $CuSO_4$ exposures, a LC50 of 0.424 mg/L was calculated (versus LC50 values ranging from 0.47-5.6 mg/L for 96-h trout bioassays; from EnviroTox database). Studies are underway to optimize transcriptomics assays from these samples using EcoToxChips and UPXome, with the ultimate goal to be able to derive transcriptomics points of departures. Taken together these results provide a foundation towards establishing a novel testing platform for chemical and environmental risk assessment.

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Rapid and efficient processing and long-term cryopreservation of brain tumour resected tissue for the culture of brain tumour explant organoids to replace animal models in brain cancer research

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

Preclinical animal models are used to test and evaluate the efficacy of drug therapies for glioblastoma. However, these animal models fail to accurately recapitulate human brain tumours' biology. Patient-derived glioblastoma explant organoids (GBOs) have recently emerged as a promising live animal-free model for studying glioblastoma. GBOs contain the human-relevant tumour microenvironment and can predict a patient's response to therapy.

Current methods to generate GBOs involve culturing glioblastoma tumour tissue pieces of ~1 mm diameter in a defined media without Matrigel. Once started, cultures are maintained for > 4 weeks until they can be long-term cryopreserved. These current procedures are time-consuming, expensive and technically demanding, which has impeded the broad adoption of this technology to replace animal models in glioblastoma research.

In this work, we developed an optimized method to generate GBOs. This method permits rapid semi-automated tumour tissue processing (< 1 hour), cryopreservation, and short/long-term storage conditions with essential laboratory equipment. This allows researchers to access tumour samples interstate or overseas and generate large GBO biobanks covering glioblastoma heterogeneity. This resource will thus facilitate systematic preclinical personalized research in a physiologically human-relevant preclinical model, thus replacing the need for animal models for brain tumour research.

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Evolving role of investigative toxicology in the pharmaceutical industry

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In recent years nonclinical toxicology evolved from a descriptive to a science driven investigative/mechanistic discipline. To obtain a detailed assessment of the adoption, range, and impact of investigative toxicology (I-Tox) in supporting drug discovery and development in the pharmaceutical industry, two surveys of 14 pharmaceutical companies were conducted several years apart. The survey results, together with case studies, reveal that I-Tox focus has evolved from supporting small molecule programs to a growing need for experimental support of newer drug modalities. Common deliverables of I-Tox include target safety assessment, hazard profiling of leads (*in silico*/*in vitro*) and in-depth hazard qualifying/quantifying experiments that support lead optimization and drug candidate selection prior to *in vivo* studies initiation. *In vitro* assays are commonly used for hepatotoxicity and cardiovascular toxicity profiling; however, there is a crucial need to improve the *in vitro* to *in vivo* translation. New technologies and associated models/assays such as organs on a chip, stem cell derived systems, advanced imaging methods, advanced molecular and cellular models, genome editing, and quantitative modelling approaches are impacting drug design, selection, and progression. During preclinical toxicity studies, I-Tox is key to understand the mode of action of toxicities observed and their relevance to humans. Furthermore, a deep understanding of the mechanism that underlies such toxicities together with an estimate of its human relevance may allow a project to continue. Finally, we emphasize the importance of collaboration between pharma, academia, service, and technology providers to further enhance our ability to discover and develop safe medicines.

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A thorough comparative study of the use of fetal calf sera, human AB sera and human platelet lysate with the focus on the cultivation of mesenchymal stromal cells

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Optimizing the expansion and differentiation of human mesenchymal stromal cells (MSCs), medium supplemented with fetal calf serum (FCS) has been widely applied. Despite ethical concerns, batch-to-batch variability, lack of reproducibility, and the risk of unknown infectious reagents in FCS or immunogenic reactions to xenogeneic proteins, FCS is still the gold standard. Human platelet lysate (hPL) and human AB serum (HS) seem to be valuable alternatives due to their human origin and their promoting effect on cell expansion. However, their sustained effect on the differentiation capacity and the immunophenotype of MSCs is still insufficiently studied.

Here, we aimed to investigate the influence of commercially available sera for FCS, hPL and HS on MSCs in terms of plastic adherence, multipotency, surface marker pattern, proliferation and metabolic activity. Furthermore, we performed a label-free quantitative proteomic analysis to identify the proteins that contribute to any difference.

A total of 255 proteins were identified in HS, 154 in hPL and 98 in FCS, with profound differences in terms of proteins displayed by metabolic processes and the cytoskeleton. We found significant differences in the differentiation ability into the osteogenic, adipogenic and chondrogenic lineage using quantitative alizarin red, oil red or Alcian blue assays. This result was confirmed by ELISA assays for adiponectin, free phosphate and GAGs Interestingly, using flow cytometry, we found profound differences in the pattern of surface markers commonly used to define MSCs.

We conclude that the choice of media supplementation has a major impact on crucial functional properties of cells such as MSCs.

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Dimensionality reduction algorithms for hybrid QSAR models of mutagenicity

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Chemical risk assessments are overly reliant on animal testing, which carries ethical, scalability and validity-based concerns. This may be alleviated by computational approaches such as Quantitative Structure-Activity Relationship (QSAR) models, which commonly use deep learning to predict toxicological properties and may be limited by "the curse of dimensionality". The aim of this study was to explore various dimensionality reduction techniques to build deep learning driven QSAR models of mutagenicity, exploring which combinations of techniques in hybrid models could give rise to the most accurate mutagenicity predictions. Knowledge graphs of Tanimoto similarity coefficients were built to quantify molecular structures from an Ames mutagenicity dataset of 11,268 molecules. Dimensionality reduction to 100 dimensions was achieved via six different methods, namely (1) Principal Component Analysis (PCA), (2) Kernel PCA, (3) Independent Component Analysis, (4) Isomap Embedding, (5) Autoencoders and (6) Locally Linear Embedding. Following this, feed-forward neural networks (with two hidden layers of 500 neurons) were used to classify mutagenic and non-mutagenic chemicals. Hybrid models were then constructed by pairing different models and only outputting mutually agreed predictions. The most optimal dimensionality reduction techniques for constructing a hybrid model were Kernel PCA and Autoencoders, resulting in an overall accuracy score of 75%, as well as 75% agreement between models. Percentage agreement between models was negatively correlated with overall accuracy score. Overall, it was concluded that the hybrid QSAR models of mutagenicity built, using different dimensionality reduction techniques, were effective in predicting mutagenicity, with non-linear techniques giving rise to the highest accuracy scores.

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Development of a predictive human stem cell-based neurosphere model for neuroinfectiological studies

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Venezuelan equine encephalitis virus (VEEV) can cause severe encephalitis with mechanisms largely unknown. Its pathogenesis has a biphasic course of replication in peripheral organs and neurons followed by infection of central nervous system (CNS) through the olfactory nerve. Maternal or early childhood VEEV infection can lead to severe neurological sequelae including cognitive deficits (< 35% under 5 years) or (fetal) death (< 10% mortality rate). Currently, no vaccine has been licensed. We aimed to establish a novel human *in vitro* neurodevelopmental model to characterize the course of infection helping to predict potential long-term neuronal damage and to identify therapeutic targets. We discovered that GFP-labeled VEEV was able to infect nearly 100% of peripheral motor neurons derived from human induced pluripotent stem cells (hiPSCs). To evaluate VEEV-induced effects in the CNS, we successfully established an infection model based on the differentiation of hiPSC-derived neurospheres. Neurospheres are 3D cell aggregates consisting of neural progenitor cells able to mimic basic processes of brain development: proliferation, migration, differentiation into neuronal and glial subtypes and network formation. In neurospheres, the virus primarily infected non-neuronal cells, likely radial glial cells, which modulate migration in the developing brain. A mass spectrometry-based characterization of neurospheres on a proteomic level revealed high abundances of various neural and neurotrophin-signaling markers and will be applied to investigate virus induced alterations in future experiments. Altogether, our results show that human neurospheres are suitable as predictive *in vitro* model system to advance our knowledge of neuronal pathogens and host interactions in human brain development.

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Endocrine-dependent, cell-specific, functional biomarkers of clinical relevance as cross cutting tools in *in vitro* toxicology: An effect-based approach to endocrine disruption

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Cell-based assays using human cell lines representative of endocrine-targeted tissues and functional biomarkers of clinical relevance have been used to identify and characterize potential Endocrine Disruptors (Lorenzetti et al., 2015, doi:10.4415/ANN_15_02_16, and refs therein). The final goal of the 3R-based project "Endocrine-dependent, cell-specific, functional biomarkers of clinical relevance as cross cutting tools in *in vitro* toxicology", granted by the Italian Ministery of Health, will be the development of a tool to perform a rapid and cost-effective hazard identification of chemicals as aimed by the European Regulation REACH (2006/1907/EC) for the identification of Substances of Very High Concern with Endocrine Disrupting properties (REACH art. 57f).

Two hormone-responsive, human-derived cell lines are used as *in vitro* model systems: LNCaP (prostate epithelium) and BeWo (trophoblast-like cells). Hormone-like chemicals (i.e., phthalates, bisphenols and parabens) are used to assess in a dose-dependent manner (1 pM - 100 microM range): i) cytotoxicity (by MTS assay), ii) PSA and beta hCG secretions (by ELISA-based assays) in LNCaP and BeWo cell culture supernatants, respectively, as endocrine-dependent, cell-specific, functional biomarkers. Obtained data will be compared with gene reporter assays (AR- and ER-CALUX) as described in the OECD TGs 458 and 455.

Data on cytotoxicity, protein secretion and CALUX-based gene reporter assays will be shown and discussed taking into accounts the specificity of mechanistic- and effect-based *in vitro* toxicological outputs. A data integration will be performed in order to merge different *in vitro* toxicological outputs in an updated AOP for the reproductive tissues of interest.

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Developing quantitative metrics for accountability in strategic roadmaps

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Strategic roadmaps to replace the use of animals with more human-relevant non-animal research and test methods provide an invaluable resource to develop consensus on pathways to work towards this goal. In these documents, government bodies and other organizations outline their programs and activities to incorporate new testing methods that predict human health or environmental outcomes without the use of animals. Notably, these roadmaps often rely on the number of initiatives to determine success; however, an increase in the number of non-animal methods or activities does not necessarily translate to a reduction in animal use. Instead, quantitative metrics measuring the effects of the development and availability of non-animal methods on the use of animals in research and testing are critical to set goals, monitor progress, and provide accountability for resources spent on initiatives. This presentation specifies a step-by-step approach to define a strategy to track the use of non-animal methods and their effects on animal use. It establishes metrics for animal savings from *in silico*, *in chemico*, and *in vitro* methods; study waivers; and intelligent design based on the utility of the data (i.e., what the data are used for and its level of certainty). Developing and incorporating metrics within roadmaps to measure the impact of non-animal methods ultimately facilitates the identification of programs that are providing the greatest reductions in animal use and those that are not having their intended impacts, and it highlights gaps where the development and/or use of non-animal methods should be prioritized.

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Mechanisms of tacrolimus-induced nephrotoxicity in proximal tubular-like cells

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Introduction: Nephrotoxicity is often observed in response to toxic compounds, which may cause severe human health problems. Optimisation and characterisation of human renal *in vitro* systems is therefore important for the development of New Approach Methods (NAMs). Here, we used the human proximal tubular cell line

RPTEC/TERT1 and proximal tubular-like (PTL) cells derived from human induced pluripotent stem cells (hiPSC) to investigate mechanism of toxicity of tacrolimus (FK506), a widely used immunosuppressive drug associated with kidney injury.

Methods: PTL and RPTEC/TERT1 were treated with different concentrations of tacrolimus to assess cytotoxicity through resazurin assays and cellular stress was measured through enhanced lactate production. P-glycoprotein (PGP) transport upon treatment was measured by calcein-AM assays. Deregulated gene expression (DEGs) after tacrolimus treatment will be analysed through genome-wide transcriptomics (RNA-Seq).

Results: PTL showed polarisation and expression of the tight junction proteins ZO-3 and occludin and proximal tubular marker megalin. Cell viability of PTL decreased to ~75% and 62% after 24 and 48 hours of tacrolimus treatment (24 µM), respectively, whereas RPTEC/TERT1 cells did not show decreased viability. Lactate production increased in both cell types after 24 hours of treatment, suggesting cellular stress. Tacrolimus also exhibited potent PGP inhibition as measured by enhanced cellular calcein retention. Currently, investigations on the effects of tacrolimus on DEGs are ongoing.

Conclusion: This study could help to unravel toxicity mechanisms of tacrolimus. The outcome of this study could also contribute to further implementation of *in vitro* methodologies for toxicity testing and replacement of animal models.

Presentation: Poster

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Cytotoxicity of pesticides exposed to rainbow trout gill, liver, and intestinal cell lines

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Each year millions of fish are used for effluent testing and chemical evaluations. The rainbow trout gill cell line (RTgill-W1) was recently standardized by the OECD as an alternative to animal model to assess the cytotoxicity of chemicals. Despite this achievement, it is not clear how responses in the gill compare to other tissues. The purpose of this study was to compare cytotoxic responses in the RTgill-W1 against responses in rainbow trout liver and intestinal cell lines. The first objective was to optimize OECD Test No. 249 to operate in a 96-well plate format, thus increasing the efficiency and scalability of the test assay. The second objective was to characterize cytotoxic responses in the three cell lines following exposure to 19 pesticides considered high priority to Canadian ecosystems and to compare the resulting *in vitro* data with LC50 results from whole animal studies. Optimization to 96-well plates did not interfere with quality control checks laid out in OECD Test No. 249 and did not affect cytotoxicity results. Cytotoxic responses varied across three cell lines with LC50s ranging from 26-500 µM in gill cells, 18-600 µM in liver cells, and 7-300 µM in gut cells. Taken together, these results show that OECD Test No. 249 (as well as studies on trout liver and gut cells) can be run on 96-well plates, preliminary analyses suggest that cytotoxic responses differ across the cell types, and that these results do not relate well with *in vivo* data, thus necessitating more research.

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Longitudinal characterisation of TK6 cells sequentially adapted to animal product-free, chemically defined culture medium: Considerations for genotoxicity studies

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In vitro approaches are an essential tool in safety screening of new molecules. Currently, FBS is routinely used as a supplement in cell culture medium, but batch-to-batch variability may introduce inconsistency in inter- and intra-lab assessments. Several chemically defined serum replacements (CDSR) have been developed to provide an alternative to FBS, but not every cell line adapts easily and successfully to CDSR-supplemented medium, and the long-term effect on cell characteristics remains uncertain.

The aim was to adapt the TK6 cells to CDSR-supplemented medium and evaluate the long-term effects on the cell viability and functionality.

Gradual and direct adaptation methodologies were compared by assessing the cell proliferation, size and viability every passage. A morphology study by high content imaging was performed and the expression of CD19 and CD20 was conducted via flow cytometry to assess the potential for phenotypic drift. Finally, functionality of cells in the OECD TG 487 assay was evaluated.

The transitioned cells showed comparable ($p > 0.05$) viability and cell size as the parent FBS-supplemented cells., Cell morphology (cellular and nuclear area, sphericity) and phenotype (CD19

and CD20 surface markers) were in line ($p > 0.05$) with the original cells. The new cells performed satisfactory in a pilot OECD TG 487 assay.

TK6 were successfully transitioned to animal product-free medium. The TK6 cells adapted to CDSR-supplemented medium shows potential to eliminate variability from FBS and increase the human relevance of *in vitro* methodologies in standard mutagenic *in vitro* assessments such as the formation of micronuclei (OECD TG 487).

Presentation: Poster

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Animal use and opportunities for reduction in carcinogenicity studies for pharmaceuticals

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For most human drugs, international guidelines require evaluating carcinogenicity to rats and mice in long-term studies. An addendum to these guidelines should decrease the number of long-term studies in both species: for mice, it prioritizes short-term studies and recognizes an exposure ratio for dose-setting; for rats, it recommends assessing the weight of evidence to determine whether a study would add value. By analyzing how animals were used in 109 carcinogenicity studies for New Drug Applications recently approved by U.S. FDA, we compared reductions achievable by implementing these and other recommendations. Replacing the remaining long-term studies in mice with short-term studies would result in a 17.3% reduction, while waiving 15 studies in rats based on histopathology in chronic studies, in a 12.9% reduction; no findings of preneoplasia predicted no findings of neoplasia with 93% accuracy. Evaluating systemic exposure by microsampling minimal blood volumes, to eliminate toxicokinetics studies, would result in an 18.7% reduction; on average, three times more mice than rats were used in both study types. Using single rather than dual negative control groups would result in another 7.8% reduction. Finally, submitting genotyping data for rasH2 mice instead of using positive control groups would use 640 fewer mice; lung adenomas were found in nearly all mice administered urethane, supporting proposals to eliminate positive control groups. Of the 19 drug labels where FDA indicated concern for carcinogenicity to humans, it was based, in part, on carcinogenicity to rats or mice for only two; for two-thirds, no carcinogenicity studies were even conducted.

Presentation: Poster

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Molecular mechanisms of heavy metals in RPTEC/TERT1 cells and iPSC derived proximal tubule like-cells

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Heavy metals, such as cadmium and arsenic can be found in food, water, air and in tobacco smoke. The half-life of some metals, e.g., cadmium can be more than 20 years and increase the risk of diseases of the kidney, lungs and bones. Both cadmium and arsenic are classified human carcinogen group 1 by IARC. Once inside the cell, free metal ions activate the metal stress response pathway increasing the production of metallothionein (MT) proteins, which chelate the metal ions.

Here, we investigated the effects of four metals in human iPSC derived proximal tubule like cells containing a GFP reporter for Nrf2 dependent gene hemeoxygenase 1 (HMOX1) and in the human renal proximal tubule cell line RPTEC/TERT1. Sodium arsenite ($NaAsO_2$), cadmium chloride ($CdCl_2$) and mercury chloride ($HgCl_2$) exposure resulted in a rapid increase in HMOX1 expression. $NaAsO_2$ was the most potent (3 µM) followed by $CdCl_2$ (10 µM) and $HgCl_2$ (30 µM). In RPTEC/TERT1 cells gene expression for MT2A and HMOX1 followed similar profiles. There was no effect of lead acetate (PbAc) on these parameters up to 90 µM over 24 h. Longer exposures of PbAc (starting at 72 hours, 30 µM) however, increased transepithelial electrical resistance in RPTEC/TERT1 cells.

In summary, of the 4 tested metals, $NaAsO_2$, $CdCl_2$, $HgCl_2$ were found to induce HMOX1, a marker of oxidative stress while $NaAsO_2$ and $CdCl_2$ also induced MT2A. More work will be required to further elucidate these results and to study the impact of exposure to MT bound metals.

Presentation: Poster

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Re-engineering the research enterprise

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To understand, prevent, treat and cure human disease, we need to shift the biomedical research paradigm – away from the use of animal models that remain poorly predictive for human safety and efficacy and toward innovative, nonanimal approaches. To achieve this, we suggest an ambitious program is adopted by research funders and propose a model for the US National Institutes of Health (NIH).

For the NIH model, we show how creation of "Science Innovation Champions" at each NIH Center and Institute could re-engineer the research enterprise to accelerate the shift away from animal use. Science Innovation Champions would have expertise in the development and use of the non-animal, new approach methodologies (NAMs) and would be charged with the advancement and implementation of NAMs across all NIH Institutes and Centers.

In this presentation, we map out a ten-year plan which aligns NIH's mission to seek fundamental knowledge about the nature and behavior of living systems and apply this to enhance health, lengthen life, and reduce illness and disability with a significant reduction in animal use. We suggest deliverable items for 2-, 5- and 10-year milestones and propose metrics that could be applied to monitor progress toward a goal of animal replacement.

Ultimately, this program would enable each NIH center and institution to undergo catalytic, transformative change of benefit to science, society and animals.

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Application of human iPSC-derived proximal tubular-like cells for transport studies

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Within the nephron, the proximal tubule is particularly vulnerable to toxicity due to the presence of numerous transporters that may lead to higher intracellular exposure levels to toxic substances. Drug-induced nephrotoxicity can contribute to acute renal injury and chronic kidney disease. The development of human renal *in vitro* models may contribute to the replacement of animal models for predicting proximal tubule injury. We have developed a protocol to differentiate induced pluripotent stem cells (iPSC) into proximal tubular-like cells (PTL) within 14 days [1]. PTL express megalin, display a polarised phenotype and show P-glycoprotein (ABCB1) transport. Further characterisation of renal transport is vital to develop a suitable model for drug discovery and toxicity studies.

Organic cation transport was studied using the renal proximal tubular epithelial cell line RPTEC/TERT1 and iPSC-derived PTL cultured on microporous growth supports. Functional transport activity was measured using the fluorescent substrate ASP (12.5 µM). Tight barrier formation was confirmed by lucifer yellow retention and transepithelial electrical resistance (TEER) measurements. In both models, ASP was taken up from the basolateral side and could be inhibited by basolateral application of quinidine (50 µM). Furthermore, apical application of cimetidine (100 µM) resulted in an intracellular accumulation of ASP, likely through interference of apical multidrug and toxin extrusion (MATE) transporters. These results show the suitability of the iPSC-derived PTL model for studying organic cation transport.

Reference

[1] Chandrasekaran, V. et al. (2021). Generation and characterization of iPSC-derived renal proximal tubule-like cells with extended stability. *Sci Rep 11*, 11575. doi:10.1038/s41598-021-89550-4

Presentation: Poster

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Brazil approaches the cosmetics animal testing ban: The argument for a federal bill

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The current patchwork of state prohibitions of cosmetic animal testing in Brazil, together with specificities of the Brazilian regulatory framework, makes a federal law necessary for practical impact. The Federal District and 12 states have local laws prohibiting these tests, each with its particularities, complicating compliance. In addition, there is currently no effective regulatory pressure for the industry to choose non-animal methods over the traditional ones. The National Council for the Control of Animal Experimentation (CONCEA) is responsible for, among other competences, accrediting institutions that use animals in education, testing and research; and establishing norms for animal use, e.g., Normative Resolutions (NRs) recognizing testing methods, including non-animal methods, with a 5-year deadline for mandatory replacement. However, this recognition process is very time consuming, and there is currently no system to monitor compliance to NRs nor to assess animal use on tests for which the replacement deadline is expired.

CONCEA could publish a NR banning animal testing for cosmetics, but the social claim to ban cosmetics animal testing would not be met, as tests may continue to be performed – either locally, as there is no monitoring system, or in countries where they are allowed – and cosmetics tested on animals would continue to be sold in Brazil.

After nearly a decade in the National Congress, the Bill 70/2014, which enacts the federal ban, passed the Senate in 2022. Passage in 2023 would finally end cosmetics animal testing and the sale of cosmetics tested on animals in Brazil.

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Safety assessment of toys and school supplies in Brazil: Moving away from animal testing

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Protection of animals used for scientific purposes and the promotion of alternatives to animals for product safety assessment is limited in Brazil. Regulations covering safety assessment are complex, administered by different agencies for different regulations. The National Council for the Control of Animal Experimentation (CONCEA) has recognized some alternatives, but there is no means of ensuring these Normative Resolutions are adopted by laboratories and agencies. The Brazilian Health Regulatory Agency (Anvisa) accepts data from alternatives, but other agencies establish the requirements for safety of specific products. For example, the safety standards for children's products such as toys and school supplies are established by the National Institute of Metrology, Standardization and Industrial Quality (Inmetro). These standards demand testing of finished products using either *in vitro* or *in vivo* methods for acute oral toxicity, skin and eye irritation, which is at odds with Brazilian law and CONCEA resolutions. HSI worked with the agencies to achieve consensus and adapt best practices applied in other countries to the regulation of school supplies in Brazil. The current strategy is now based on information on the ingredients, and the use of animals is allowed only as a last resort and with technical justification.

This was an important advancement in the school supplies sector, and now HSI is engaging Brazilian stakeholders to review toys regulations, aiming to take a similar approach. This is a concrete example of working with agencies to leverage scientific advances used in one region or sector to make progress in another.

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A new barrier-on-chip system for one-fits-all organ modelling: The example of the gut

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To overcome the challenges and ethical concerns linked to animal testing, efforts from industry and academia are focused on finding alternatives to better simulate complex human physiology while improving predictivity and translation.

In this work, we present the AXBarrier-on-Chip system, a platform enabling to tune strain parameters to reproduce organ-specific mechanical cues (gut, skin, lung, etc.); or strain-related (patho) physiological processes. By resting at the ultra-thin, porous, and soft cell culture substrate of the AX12 plate, cells experience near-physiological conditions and preserve specific phenotypes.

To investigate the applications of this platform, we aimed at replicating the gut environment with 3D peristalsis. For that, a co-culture of cell lines representing enterocyte and goblet features (Caco-2 and HT29) was established and exposed to 3D mechanical stimulation following typical day activity patterns (active – day/resting – night).

The newly generated gut-on-chip model exhibited cell polarization (brush border formation), expression of typical gut markers, stable barrier formation, and permeability values within physiological ranges. Further, we confirmed that 3D peristalsis enhanced the sensitivity to proinflammatory stimuli, leading to a pronounced barrier disruption and secretion of proinflammatory mediators.

Our results support the versatility of the AXBarrier-on-Chip system for organ modelling with specific mechanical demands. As illustrated by the gut-on-chip model developed in this study, the implementation of physiologically relevant features has an impact on *in vivo*-like simulation. This increased accuracy and human relevance are essential for model's predictivity, which could have a significant contribution to decision-making and shortening drug development timeframes by reducing animal testing.

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A lung-on-chip platform to assess *in vitro* safety and toxicology with physiologically relevant outcomes

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Classically, the approval of new products in the consumer goods and pharma industries required a thorough safety evaluation involving animal testing. Thanks to the development of advanced *in vitro* models and *in silico* approaches, now regulatory agencies contemplate the possibility of human-relevant testing bypassing animal experimentation.

Here, we present an approach for lung modeling including key physiological parameters, the AXLung-on-chip system. We explored the relevance of our model through different case studies in the context of toxicology, drug safety and efficacy.

To assess the exposure to environmental hazards, an alveolar model was exposed to nebulized inhalants (Cloudα AX12). Integrating breathing dynamics increased model's sensitivity to nebulized nanoparticles commonly used in the cosmetic and food industry (ZnO and TiO_2), and toxicants such as PHMG. Exposure to these products incited pro-inflammatory responses accompanied by an increased cytotoxicity (> 2.5-fold) and barrier breakdown (> 3-fold decrease).

Further, by using patient-derived models, we evaluated the safety of an oncologic drug and the efficacy of an antifibrotic compound. Our immunocompetent barrier model reproduced specific signatures associated with increased inflammation and immune cell activation, relevant hallmarks for preclinical safety evaluation. Additionally, we proved the antifibrotic effect of the compound *in vitro*, which reverted collagen secretion in the efficacy model.

In summary, our results underline the relevance of the AXLung-on-chip system for molecule testing. Its significant impact in decision making and drug development is further supported by regulatory submissions. Overall, we believe this technology promises to alleviate the burden on animal testing by increasing *in vitro* predictivity and accuracy.

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Cell-based reporter assays for nuclear receptors as predictive tools in reproductive and developmental toxicology

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Reproductive and developmental toxicology is an important area of research, as exposure to toxicants during critical periods of development can have long-lasting effects on health and well-being. Traditional animal-based tests for reproductive and developmental toxicity are costly, time-consuming, and involve the use of a large number of animals. There is growing interest in developing alternative methods that are more predictive, efficient, and ethically sound. In this context, cell-based reporter assays for nuclear receptors are emerging as powerful tools for evaluating the effects of chemicals, alone or part of complex mixtures, on reproductive and developmental processes. Nuclear receptors, including estrogen receptors, androgen receptor, and thyroid hormone receptor, are transcription factors that play key roles in regulating reproductive and developmental processes. These receptors can be activated or inhibited by a wide range of chemicals, including environmental contaminants and pharmaceuticals, leading to endocrine disruption and adverse effects on fertility, pregnancy outcomes, and offspring development. Cell-based reporter assays allow the detection of the binding of chemicals to nuclear receptors, the induction or inhibition of receptor-mediated transcriptional activity, and the assessment of downstream effects on cellular functions. Recent advances in the development and application of cell-based reporter assays for nuclear receptors in reproductive and developmental toxicology will be discussed highlighting their sensitivity, specificity, reproducibility, and throughput, and their potential for predicting adverse outcomes in humans and other species. We will also discuss the challenges and opportunities for integrating these assays into regulatory frameworks for chemical safety assessment, environmental monitoring, and regulatory decision-making.

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A 3D human cell-based *in vitro* model for chronic *Pseudomonas aeruginosa* lung infection

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The opportunistic human pathogen *Pseudomonas aeruginosa* causes chronic and lethal lung infections in susceptible individuals such as COPD and cystic fibrosis patients. Despite ongoing research, host-pathogen interaction studies are limited by the lack of suitable long-term infection models. Without external aids such as agar beads, bacteria are rapidly cleared from the lungs of infected animals and the existing *in vitro* models are usually confined to short time periods due to high bacterial cytotoxicity.

To overcome this problem, we established a new system for modelling chronic *P. aeruginosa* infection using both primary (normal bronchial epithelial) cells as well as a lung epithelial cell line (Calu-3). Cells were grown at the air liquid interface followed by infection with well characterized, patient-adapted clinical isolates from the BACTOME database.

Bacterial load, cell viability, pro-inflammatory cytokine secretion and gene expression were analysed between 6 hours and 5 days after infection. Infected cells maintained a viability of up to 95% despite high bacterial loads (10^8 CFU/ml) and elevated immune markers (including IL-1β, IL6, IL-8 & G-CSF) up to 5 days post-infection. Concurrently, H&E staining showed an intact polarized monolayer throughout infection and bacterial transcriptome analysis revealed upregulation of virulence factors such as LPS and the type III secretion system.

We propose this 3D human cell model to be used for future host-pathogen studies and compound screenings as a physiologically relevant alternative to standard antimicrobial testing. The described method can be performed in a basic laboratory setting and allows for detailed monitoring of cellular and bacterial parameters.

Presentation: Poster

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Implementation of a human cell-based malaria-on-a-chip phenotypic disease model for drug efficacy evaluation

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Of all the *Plasmodium* spp. found in humans, the infectious protozoans responsible for malaria, the falciparum species is the most fatal. The need for a human-based platform to model disease pathophysiology and monitor drug efficacy is much needed for preclinical drug development. While NHPs have been conducive in breakthrough malarial discoveries, using NHPs as animal models is expensive and has limited availability. In this study a human-based Malaria-on-a-Chip model was developed to further evaluate drug efficacy and off-target toxicity of common antimalarial therapeutics: chloroquine, lumefantrine, or artesunate. Parasite clearance times, viability of all organ constructs, as well as hepatic metabolic activity and splenic immune response were monitored over the course of infection and treatment. Following administration of treatment, a dose dependent clearance of the parasite was observed in both strains for all compounds. Recrudescence of the 3D7 strain was observed by day 7 for all chloroquine and lumefantrine treatments while recrudescence was not observed by day 7 with artesunate treatment. W2, a chloroquine resistant strain, infected systems exhibited a stabilization of parasitemia levels by day 7 when treated with chloroquine and lumefantrine, while those treated with artesunate continued to diminish. A significant dose dependent effect in treated organ viability was observed with chloroquine and artesunate treatment, but no significant changes were observed with lumefantrine treatment. With limited animal model resources involving human malarial infections, the data from the Malaria-on-a-chip model offers a human-based approach to model pathophysiological pathways and pharmacokinetic/pharmacodynamic models.

Presentation: Poster

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Efficacy of bacterial lysate OM-85 in RV1b-infected murine lung tissue slices

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Rhinovirus (RV) is the main cause of common cold and major risk factor of exacerbations in chronic respiratory diseases. RV infections in mouse models are cleared very quickly, thus only mild to moderate infections are represented. From the 3R perspective, to overcoming this, *ex vivo* models such as precision-cut lung slices (PCLS) can be used. OM-85 is a soluble lysate of bacteria isolated from the respiratory tract and used to prevent respiratory tract infections. This dataset presents OM-85 effects against viral infection on murine PCLS.

PCLS were prepared from agarose-filled Balb/c lungs, treated prophylactically with OM-85, infected with RV1b, and post-treated with OM-85 for up to two days. Immune changes induced by viral infection and OM-85 treatment were analyzed.

Ex vivo RV1b-infected murine PCLS induced IL-6, IP-10 and IFN-α secretion. Pretreatment with OM-85 resulted in modulation of several immune mediators. Transcriptomic analysis confirmed a strong immunomodulatory effect of OM-85 in RV1b-infected and uninfected PCLS. Transcriptomics showed that OM-85 alone induced differentially expressed genes (DEGs) and higher numbers of DEGs in presence of RV. Principal Component Analysis revealed clear clusters indicative of product, viral-dependent and -independent changes induced by OM-85. Treatment and infection of PCLS showed early points of immunomodulation that can model the immune response.

The results showed a very prominent immune host effect of OM-85 alone and additionally to the RV induced immune response in murine PCLS. OM-85 could lead to a priming of immune cells in 3R-conform model PCLS and facilitate a more efficient clearance of viral infections.

Presentation: Poster

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Determining the impact of metal oxide nanoparticle size and solubility on lung epithelial cell toxicity

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Metal oxide nanoparticles (MONPs) are one of the most commonly used types of engineered nanomaterials (manufactured substances with at least one dimension in the 1-100 nm range), and they are used in a wide variety of applications in several industries. Many studies have observed cytotoxicity, genotoxicity, oxidative stress, and pulmonary inflammation induced by MONPs in *in vitro* and *in vivo* models. The overall objective of this study was to investigate the cytotoxic and genotoxic potential of metal oxide nanoparticles (MONPs) of different size and solubility to provide insight into the importance of physicochemical properties on MONP induced toxicity. FE1 mouse lung epithelial cells were exposed to manganese dioxide (MnO_2) and iron (III) oxide (Fe_2O_3) nanoparticles (NPs), microparticles (MPs), and soluble manganese sulfate ($MnSO_4$). Cell viability was assessed after 24 h and 48 h using the trypan blue exclusion assay while DNA damage was assessed using the high-throughput CometChip® platform after 2 h and 4 h of exposure. Changes in gene expression after 24 h and 48 h were examined using microarray. The NPs in general appeared to be slightly more cytotoxic and genotoxic than the MPs. MnO_2 NPs were more potent than Fe_2O_3 NPs with respect to genotoxicity. Dissolved manganese ions did not induce toxicity. These results suggest that size and chemical composition influence their potential to induce toxicity. The information produced will address data gaps identified by health risk assessors and aid in the development of read-across strategies for the assessment of other MONPs.

Presentation: Poster

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Extrapolation of *in vitro* bioactivity and toxicity data to relevant human exposures

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In vitro bioactivity and toxicity data are increasingly being considered to facilitate hazard and risk assessment. One prominent example is the calculation of Administered Equivalent Doses (AEDs). AEDs are typically calculated as the ratio of an *in vitro* POD (e.g., AC50, µM) and the estimated steady-state blood or plasma concentration (CSS, µM) corresponding to an oral dose of 1 mg/kg/d (e.g., AED = AC50/CSS 1 mg/kg/d). A key underlying assumption inherent to the calculation of AEDs is that the *in vitro* POD (e.g., AC50) and steady-state blood or plasma concentration are directly comparable exposure metrics. This assumption is problematic for several reasons stemming from the fact that *in vitro* PODs are almost always reported on a nominal basis. Because the composition of assay medium is different from the composition of plasma and blood and other losses can occur *in vitro* (e.g., volatilization), there may be substantial "mismatches" between *in vitro* PODs and CSS with respect to the relevant exposure. To assess the potential bias in typical AED calculations, we applied an *in vitro* mass balance model (IV-MBM v2.1) to a set of organic chemicals spanning a wide range of partitioning properties across various test conditions. Differences in AEDs using nominal doses and model output can be as large as two orders of magnitude. The results clearly demonstrate the importance of considering *in vitro* disposition and bioavailability issues when extrapolating *in vitro* bioactivity data to AEDs and then comparing to them to traditional *in vivo* PODs and exposure estimates.

Presentation: Poster

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New approaches to classifying and analyzing literature for mechanistic information

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Mechanistic information is increasingly prominent in prioritizing research and risk assessment activities and for reducing the use of vertebrate animals in toxicology research. However, there is a dearth of validated process for classifying mechanistic data. Three novel classification approaches utilizing keyword searches and artificial intelligence (AI)-based named entity recognition (NER) algorithms were tested using a publicly available literature database of 120 studies related to Bisphenol A (BPA) toxicity. The first approach employs a keyword search strategy to classify literature into 19 mechanistic categories using 7,626 keywords manually curated from public databases and expert input. The BPA literature mapped to 17 of the 19 categories, with the highest percentage of studies matching categories validated by manual screening (e.g., cell signaling). The approach also identified categories not matched by manual review (e.g., protein degradation). False positive (0-4%) and negative rates (0-11%) were low and varied by category. The second approach employs a keyword search strategy to map the literature to Adverse Outcome Pathways (AOPS). This method identified 5 potential AOP matches, of which 2 were relevant. Modulation of match thresholds and removal of nonspecific entities improved identification of relevant matches. The AI-based NER method identified 46 novel (of 157 total) co-occurrences between endpoint entities and initiating or key event entities in the same sentence, that may facilitate AOP development to generate hypotheses, prioritize targets for validation, and explore key event relationships. These methods can be customized and provide rapid and unbiased approaches to classifying literature relevant to understanding disease mechanisms.

Presentation: Poster

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Addressing applicability domain and uncertainty in high throughput toxicokinetic data and applications

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New Approach Methodologies (NAMs) such as *in vitro-in vivo* extrapolation (IVIVE) and high-throughput toxicokinetic (HTTK) modeling can be used to obtain data necessary for chemical priority setting, screening, and risk assessment. However, there is a need to systematically examine existing data and NAMs to foster confidence in their application by establishing the applicability domains (AD) of available HTTK models and databases.

This presentation determines the AD and assesses the uncertainty of current IVIVE and HTTK data to estimate administered equivalent doses (AEDs) from *in vitro* bioactivity test data.

Different *in silico* approaches are applied to calculate toxicokinetic parameters and evaluate the chemical space that can be covered by current HTTK models. The comparison includes: i) Quantitative Structure Activity Relationship (QSAR) models to directly estimate total elimination half-life and a composition model, where tissue partitioning is a function of the composition of the tissue, to calculate volume of distribution ii) a generic one-compartment physiologically based toxicokinetic (1Co-PBTK) model that can be parameterized to different mammals as implemented in the Exposure And Safety Estimation (EAS-E) Suite platform, iii) the "3 Compartment steady state" model developed by the US-EPA as implemented in the "httk" R package and accounting for metabolism on the basis of *in vitro* biotransformation data. The results of this work generally support the computational IVIVE and HTTK methods required to estimate AEDs. General guidance and recommendations are provided for applying HTTK methods for AED calculations and improving HTTK modelling and risk estimation.

Presentation: Poster

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Tiered methods for bioaccumulation assessment to reduce animal testing

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Bioaccumulation (B) assessments need to be conducted as a part of chemical regulatory programs. Due to the myriad of metrics and categorization criteria and the underlying uncertainty in measured or modeled data, B assessments can be complex thus decision-making can be challenging. Tiered methods and weight of evidence (WoE) approaches are recommended to address uncertainty.

Here, we present the Bioaccumulation Estimation Tool (BET) and Bioaccumulation Assessment Tool (BAT), two complementary tools that form the basis of a tiered method to reduce unnecessary animal testing. The BET is a screening-level modelling system integrated in the freely available on-line Exposure And Safety Estimation (EAS-E) Suite platform (www.eas-e-suite.com). The BET includes mass balance bioaccumulation models for a range of representative ecological receptors (plants, invertebrates, fish, birds, and mammals), food webs and typical laboratory test animals, i.e., fish and rat. The tool is automatically parameterized in EAS-E Suite and calculates B metrics such as Bioconcentration Factors (BCFs), Bioaccumulation Factors (BAFs) and Biomagnification Factors (BMFs). The BAT is a higher-tiered tool which guides the collection, generation, evaluation, and integration of various lines of evidence (*in silico*, *in vitro*, lab and field studies) for a WoE approach for definitive B assessment decision-making.

The BET and the BAT are freely available and can readily be used by interested stakeholders from academia, industry, and the regulatory community. These tools provide a consistent and transparent framework to address uncertainty in B assessment and are envisaged to evolve with scientific and regulatory developments.

Presentation: Poster

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3D-bioprinting brain model: New platforms for brain disease modeling and drug screening

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Our current understanding of brain physiology and pathology is still limited by the lack of adequate, predictive, and inclusive models that account for the complex multi-layer architecture of the human brain. Through recent technological advances, tissue engineering is achieving functional 3D models that provide accurate cell-to-cell and cell-to-matrix interactions. In the present work, we combine 3D-bioprinting technology with induced pluripotent stem cells (iPSCs) derived neurons and astrocytes to generate personalized 3D neural tissue models suitable for brain disease modeling and drug screening. We first assessed the feasibility of our print settings on two natural and two synthetic bioinks used for neuronal tissue engineering. Secondly, we incorporated fluorescent neuroblastoma SHSY5Y-RFP and astrocyte-GFP cell lines to compare cell morphology and viability with different bioinks. Finally, we selected the bioinks with superior cellular responses and printability and used them in combination with iPSCs-derived neuronal precursor cells (NPCs) and astrocytes. Our preliminary results show better cellular response with natural bioinks, long-lasting cellular viability, and neurite spreading and elongation for up to one month in culture. Overall, our data indicate the potential of personalized 3D-bioprinted neuronal models as future possible drug screening and disease modeling platforms.

Presentation: Poster

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Pharmacokinetic / pharmacodynamic modeling of microphysiological human-on-a-chip systems to reduce pharmaceutical animal testing

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Hesperos, Inc. aims to substantially reduce animal pharmaceutical testing and improve compound efficiency in preclinical trials to reduce late-stage drug failure by utilizing body-on-a-chip technology with human tissues coupled to bioMEMS devices to form Human-on-a-Chip®. This technology aims to recapitulate *in-vivo* physiological markers and outputs in an *in-vitro* system to reduce cost, improve pharmaceutical tests and improve patient outcomes by investigating drug compound efficacy, toxicity, and mechanism of action without animals. Hesperos has developed a portfolio of serum-free and low volume multi-organ microfluidic systems that allow for non-invasive electrical and mechanical functional measurements on cardiac, neuronal, skeletal muscle, neuromuscular, and other tissues including functional barriers. This platform offers the ability to imitate a drug's metabolic lifecycle (pharmacokinetics) and functional effects in the human body (pharmacodynamics) to conduct toxicology and efficacy studies for disease treatment. Toward this goal, Hesperos's multi-organ microfluidic systems feature recirculation of a low volume of medium using a pumpless design, enabling pharmacokinetic studies and organ-organ interaction. A major component in the engineering design of these microfluidic systems is the application of computational techniques and pharmacokinetic modeling strategies to predict drug behavior in these devices. A combination of computational fluid dynamics (CFD) and other numerical methods is used to engineer flow rates, shear stresses, and pharmacokinetics including first pass metabolism and mixing rates using a multitude of flow schemes with dynamic rocking frequency, tilt angles and orientation. This procedure allows for device extension to multi-organ, multi-level systems while minimizing development costs for use with predictive modeling.

Presentation: Poster

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Animal-free cell culture: Toward humanized skin model

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The development of alternative methods to animal tests was a milestone in the life sciences field. It allowed us to fulfill ethical and scientific concerns, leading to advances at speed never seen before at a lower cost. However, most still rely on animal-derived components, which also raises concerns.

We explored different protocols to adapt and maintain epithelial cells to an animal-free condition and to develop a reconstructed skin model using these cells. For that, fibroblasts were cultivated in DMEM supplemented with fetal bovine serum (Dfbs) (control) or human platelet lysate (Dhpl) To support further cell attachment, a coating with collagen IV (human) was evaluated. Finally, two protocols were employed: direct change (100% Dfbs to 100% Dhpl) or gradual change with increasing concentrations of Dhpl. Direct replacement, with or without protein coating, as well as gradual change without coating resulted in impaired adhesion. The fibroblasts that underwent the gradual change associated with collagen IV coating maintained the morphological characteristics, equivalent proliferation rate, and cell viability. A reconstructed skin model generated with the adapted fibroblasts and animal product-free media resulted in equivalent morphological characteristics (thickness, number of epidermal layers) compared to tissue obtained with traditional protocol (serum dependent).

These results demonstrate that we can fully grow human primary cells in animal-free conditions. Moving forward, we will include animal-free keratinocytes in our skin model and replace animal-derived dermal matrix by polymeric or human recombinant collagen I to generate an animal-free human skin model.

Presentation: Poster

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Adaptation of a reconstituted human ocular epithelium model (ToxIn Ocular) to an animal-free condition

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The physiological characteristic of the human corneal epithelium makes it more susceptible to tissue damage caused by substances or environmental conditions. As such, eye corrosion and irritation are important endpoints for the toxicological evaluation of ingredients and cosmetics. For a long time, these outcomes have been evaluated using animal models. However, social pressure, ethical concerns, and scientific motivations lead to the development of alternative methods, such as reconstituted human corneal epithelium (RhCE) models. This project aims to develop and validate a new accessible and easily reproducible RhCE protocol based on the ToxIn Ocular model, using a chemically defined culture media and animal-free conditions. For that, HaCaT keratinocytes were seeded in 24-well inserts previously coated with collagen I (bovine) at a density of 5×10^5 cells/insert. The tissues were maintained at an air-liquid interface for 6 days until adequate stratification and two media conditions were evaluated (standard media and animal-free media). Histological analysis and preliminary irritation tests were performed to evaluate tissue morphology and functionality. The two media conditions showed no significant histological differences with 4-5 layers of cells formed, which is comparable to the human epithelium cornea. Preliminary tests also demonstrated that the model can correctly predict the irritancy potential of chemicals. With that, we have demonstrated the possibility of developing a biomimetic RhCE model using a media of known composition and animal product-free. Moving forward, we will replace the collagen I matrix with a human or recombinant source, perform biomarkers characterization, and validate the protocol for eye irritation evaluation.

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A new approach methodology for predictive DART with a C. *elegans*-based assay

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C. elegans has been of great interest to the toxicology community for years as an alternative to animal testing. It has a short life cycle, high genetic homology with humans, shares many toxicology-relevant cellular pathways, and can be cultured rapidly at a low cost. However, high-throughput developmental and reproductive toxicology (DART) studies have previously been restricted to gross phenotypes such as body size, which limits the sensitivity, specificity, and predictivity of the assay. Here we present a microfluidics-based high-throughput imaging platform (vivoScreen) that can simultaneously immobilize ~1,000 nematodes and acquire high-resolution time-lapse images of in-utero embryos from 24 individual populations per chip in 30 minutes. We analyzed time-lapse images of *C. elegans* exposed to several doses of ecotoxicology-relevant chemicals (triphenyl phosphate, piperazine, and methylmercury) and quantified *in vivo* endpoints that are relevant for DART assessments. We demonstrated that our assay was repeatable across individual technical replicates and reproducible over multiple independent experiments. Using multiparametric analyses, we were better at identifying low LOAEL values, especially with the late-stage developing embryo phenotype that was 2-10× more sensitive than the total embryo count for piperazine. Comparison of LOAELs for our sample toxicants with those from rodents and other model organisms showed that our assay correlates highly with the values obtained in rodents (Spearman correlation). These results demonstrate the utility of our assay as a New Approach Methodology (NAM) for DART, providing rapid predictive ethical *in vivo* toxicology testing without using vertebrate animals, at a fraction of the cost and time of mammalian studies.

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Mechanical stress affects the metabolism of human fibroblasts and contributes to synovial fibrosis

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Fibrosis, typical of osteoarthritis (OA), is characterized by persistent and exaggerated activation of fibroblasts due to chronic tissue injury, mechanical stress, and low-grade inflammation. Mechanical cues are important homeostatic factors in the joint, but also critical for fibroblast activation and transition from fibroblasts to myofibroblasts in fibrosis. Nevertheless, the effects of mechanical loading on OA progression remain widely unknown. Current preclinical approaches to study OA rely on *in vivo* model systems that do not enable precise control of microenvironmental cues, namely biomechanical load or shear stress.

We aimed to provide an animal-free, human-based toolset to investigate the mechanisms induced by (patho)physiological mechanical stimuli applied in a defined manner to fibroblast-like synoviocytes (FLS) in a 3D synovial membrane model.

The model is based on FLS from OA patients cultured in animal-free synthetic hydrogel for 14 days and subsequently transferred to a bioreactor chamber. This model was subjected to e.g., 0 and 5 dyn/cm² fluidic shear stress for up to 72 hours at 1.5-hour perfusion and 6.5-hour static intervals, corresponding to (patho) physiological conditions of locomotion. Mechanical forces of 5 dyn/cm² are sufficient to induce the expression of pro-fibrotic markers such as alpha-smooth muscle actin (α-SMA) and collagen type 1, compared with 0 dyn/cm². Moreover, α-SMA-positive FLS within the 3D construct were found to overexpress the metabolic key enzyme pyruvate dehydrogenase kinase 3.

Our toolset allows to study the effects of mechanical stimuli *in vitro* in a highly controlled and reproducible fashion, enhancing our understanding of initial key events of this devastating disease.

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Integrated multi-technique chemical hazard screening: A novel new approach method using the zebrafish (*Danio rerio*) larvae model and transcriptomics

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Chemicals play a crucial role in our daily lives, but they can also pose significant risks, especially with long-term exposure. Traditional animal-based methods for toxicity testing are expensive, time-consuming, unethical, and often don't provide significant insights into the underlying causes of adverse health effects. Therefore, there is a need for faster, more cost-effective, and more informative New Approach Methodologies (NAMs) to assess the ecotoxicological consequences of toxic substances on animals. We propose an integrated NAM that uses the zebrafish embryo/larvae model and four techniques to evaluate the hazards of various toxic substances: transcriptomic dose-response modelling, the metabolic energy expenditure (alamarBlue™) assay, the behavioural light-dark photomotor response assay, and the classical fish embryo toxicity (FET) test. The novel aspect of this NAM is that it allows for the comparison of different points of departure (PODs) from the four techniques to screen the overall hazard of toxic substances and identify different toxicological effects. The preliminary results show that the transcriptomic dose-response modelling technique is the most sensitive and informative technique for chemical hazard identification because it captures the majority of sub-phenotypic adverse effects, followed by the behavioural photomotor response assay which is sensitive to neurotoxic phenotypes. With further refinement and validation, this integrated NAM has the potential to become an accessible and widely adopted screening tool to assess the hazard of toxic substances that threaten vulnerable species and the environment, while also providing insights into various mechanisms of action.

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Deriving a point of departure for skin sensitization risk assessment of fragrance ingredients based on OECD *in vitro* methods

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Several *in vitro* methods addressing the first 3 key events of the adverse outcome pathway (AOP) for skin sensitization have been validated by the Organization for Economic Co-operation and Development (OECD). These methods can be combined in Defined Approaches (DA), as described in OECD guideline No. 497, to determine hazard and potency classification for regulatory purposes. Additionally, quantitative regression models based on *in vitro* methods have recently been developed to calculate a point of departure (PoD) for skin sensitization risk assessment. These models calculate a PoD as a predicted Local Lymph Node Assay (LLNA) EC3 value, using data from the kinetic Direct Peptide Reactivity Assay (kDPRA), the KeratinoSens™ (KS) assay and the human Cell Line Activation Test (h-CLAT), covering key events 1, 2, and 3, respectively, of the skin sensitization AOP. In this study, 60 fragrance ingredients, including 49 sensitizers and 11 non-sensitizers were tested in the kDPRA. These data were then combined with pre-existing KS and h-CLAT data in quantitative regression models to predict LLNA EC3 values as PoDs. Predicted EC3s were then compared to historical experimental EC3s and the No Expected Skin Sensitization Induction Levels (NESILs) in humans. For most of the fragrance ingredients in this dataset, the predicted PoDs were within or close to the area of the variation of the historical LLNA data and human NESILs. Additionally, there was minimal variability in results between models combining the 3 *in vitro* assays, resulting in a median range of fold-changes between models of 1.1.

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Advancing an ultraplexed RNA sequencing platform for high throughput gene expression analysis in ecotoxicology

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There has been increased interest in high quality, low-cost technologies to understand transcriptomic changes in non-human species such as fish, invertebrates, and plants/algae, which can serve as a foundation for the next generation of ecological toxicity tests for chemical safety evaluation. Within the context of the U.S. Environment Protection Agency (US EPA) Technology Advancing Rapid Gene Expression based Testing (TARGET) competition, the objective here was to evaluate differential gene expression in the transcriptome of four species, viz. *Pimephelas promelas* (*P. promelas*), *Daphnia magna* (*D. magna*), *Chironomous dilutus ´´*(*C. dilutus*) and *Raphidocelis subcapitata* (*R. subcapitata*) using the UPXome ultraplex library preparation technology for RNA Sequencing from Qiagen. Species were exposed to eight chemicals in microplate formats. RNA was isolated from samples exposed to the highest concentration and pooled in triplicate for each species. Libraries were prepared and checked for quality on a Bioanalyzer and sequenced on an Illumina NextSeq500. Raw fastq files were demultiplexed using Qiagen's GeneGlobe RNA Seq portal and mapped using hisat2 (*P. promelas*) and salmon pseudoalignment (other species). Further downstream gene expression analysis was performed using the Limma package on www.expressanalyst.ca. There were 4 differentially expressed genes (DEGs; adjusted p-value < 0.05) in *P. promelas* including transmembrane protein 9 and protein-cysteine N-palmitoyltransferase HHAT like protein, and 4 DEGs in *C. dilutus* including serine threonine-kinase N isoform X2 and cellular repressor of E1A-stimulated genes. No DEGs were observed in *D. magna* or *R. subcapitata*. This work demonstrates the utility of the ultraplexed library preparation technology which is scalable for high throughput gene expression analysis in species of ecological relevance.

Presentation: Poster

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Two case studies: Predicting dermal and inhalation exposure from mixtures

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Many commonly used consumer and industrial products are mixtures. Evaluation of human exposure requires knowledge of a substance's chemical properties and components of the mixture can change the effective properties of the chemical of interest in the mixture. It is desirable to be able to confidently predict the properties of constituents of interest in a mixture to save time and money and to avoid the environmental impacts of carrying out experimental procedures. We developed a new tiered system for estimating the properties of known constituents in defined mixtures. The framework is freely accessible in the Exposure And Safety Estimation (EAS-E Suite; www.eas-e-suite.com) on-line platform. Here we apply this framework as two case studies for predicting human exposure of substances originating from a mixture source: inhalation exposure of diesel fuel and dermal exposure of a transdermal drug from a simple skin cream-type product. Physicochemical properties including vapour pressures, partition ratios, and dermal permeation coefficients are predicted using different models and compared. Predictions are used to parameterize the RAIDAR-ICE exposure model to compare the variability in predicted exposure concentrations from using each model. Results show that while it did not make much of a difference whether mixture effects were accounted for in the diesel mixture, the skin cream-type mixture showed a large variability in exposure concentrations, up to a factor difference of 130 between predicted and measured values. Finally, a sensitivity analysis demonstrates how partitioning behaviour changes due to mixture effects for each of these mixtures which are very different in nature.

Presentation: Poster

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Synaptogenesis assay for developmental neurotoxicity testing in a human 3D brain model

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The rapid increase in the prevalence of many neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD), is a major public health concern, with exposures to environmental chemicals a hypothesized source of this increase. While currently available animal models of NDDs are useful tools to study disease mechanisms and phenotypes, they are low-throughput and expensive. The high costs, ethical concerns, and arguable relevance of current *in vivo* assays are prohibitive for routine chemical screening for developmental neurotoxicity (DNT). Thus, there is a critical need for robust human-relevant and fast-acting assays to replace animal tests and quickly assess the DNT of chemicals. We used 3D brain organoids to close this gap.

Using CRISPR/Cas9 genomic editing, we have introduced a green fluorescent protein (GFP) to tag a pre-synaptic protein, synaptophysin (SYP), in an induced pluripotent stem cell (iPSC) line. After quality control steps, this reporter line was differentiated into brain organoids. We then followed the expression and colocalization of the GFP tag with synaptic markers over time and quantified GFP expression upon exposure the environmental chemicals (Lead, Arsenic, Cadmium, Chromium as well as mixture of the four), which are suggested to perturb neurodevelopment and synaptogenesis, the formation and maintenance of synaptic connections. Tracking these changes over time allows for the characterization of DNT outcomes from chemical exposures.

Thus, development of this alternative *in vitro* assay could reduce the burden of animal testing in DNT screening while providing high quality, high-throughput, and relevant data for chemical risk assessment to improve human health outcomes.

Presentation: Poster

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The organ-on-a-chip: A new technology for preclinical trials

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The scientific validity of non-human animal models in predicting human toxicity in preclinical pharmaceutical testing is being questioned.

Some alternative methods have been developed to reduce the use of animals in preclinical testing. For example, animal testing is being replaced by organ-on-a-chip (OOAC) technology.

OOACs are microphysiological systems that mimic the structure and function of human organs such as the lungs, heart, and liver. They contain hollow microfluidic channels lined with living human cells and an interface that lines the inner surface of blood and lymphatic vessels. The OOAC can be coated with live human cells for drug testing, disease modeling and personalized medicine.

The OOAC can reduce the risk of drug development failures and time to market, which means faster access to new treatments and significant cost reductions.

The objective of this study is to identify what the mainly companies, areas, inventors are doing in R&D with OOAC.

The patent research was made by using Orbit/Questel and the result was 862 documents. The mainly applicants are Emulate, Harvard College and MIT. The inventors were Donald Ingber and Daniel Levner. The areas were biotechnology and chemical engineer.

With we can propose collaboration or TT with some of the applicants to improve research and products related to OOAC in Brazil.

Presentation: Poster

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OECD workshop consensus report: Ethical considerations with the use of human serum in OECD Test Guidelines

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The ethical needs and concerns with use and sourcing of human materials, particularly serum, in OECD *in vitro* test guidelines were explored in a dedicated international workshop held in 2019. The health-related aspects of the donation procedure, including tissue screening, donor health, laboratory work health protection, permission from the donor for commercial use, payment of the donors and the potential for exploitation of low-income populations and data protection of the donors; supply, availability, and competition with clinical needs; traceability of the serum and auditability/GLP needs for the Test Guideline Programme, were examined. Here we provide the recommendations of the workshop with respect to the use of human serum, and potentially other human reagents, specifically with regard to test method development for OECD Test Guideline utility as part of the Mutual Acceptance of Data requirement across all OECD member countries. These include informed donor consent terminology, a checklist of human serum information requirements to be included with the Good Laboratory Practise report, and suitable sources for human serum to ensure waste supplies are used, that can no longer be used for medical purposes, ensuring no competition of supply for essential medical use.

Presentation: Poster

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Read-across-based prediction for the non-genotoxic carcinogenicity of chemicals using molecular descriptors and *in vitro* assays

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The aim of this study is to develop an alternative method to rat carcinogenicity tests based on a read-across method using molecular descriptors as representatives of physicochemical properties and the results of mechanism-based *in vitro* assays. The results of 2-year rat carcinogenicity tests of non-genotoxic agrochemicals were collected using Risk Assessment Reports of pesticides published by the Food Safety Commission of Japan, and 80 carcinogens that induced benign and/or malignant tumors in the liver, thyroid, testis, uterus, ovary, breast, nasal cavity, stomach, and/or bladder/urethra and 46 non-carcinogens that showed no carcinogenicity in these organs were selected. These substances were then subjected to various *in vitro* assays, including HepG2 cell-based cytotoxicity tests and reporter assays of several nuclear receptors. Fisher's exact test demonstrated that there were significant associations between thyroid tumor and PXR activation, testicular tumor and AHR activation, stomach tumor and changes in cellular GSH levels, and bladder/urethra tumor and AHR activation. Then, read-across prediction for these 4 types of tumors was performed using neighborhood (source) substances selected based on Euclidean distances between substances calculated using alvaDesc molecular descriptors, with or without the secondary selection of the source substances by *in vitro* assay data. The results indicated that read-across with the secondary selection based on the assay data gave better prediction accuracy than read-across by descriptors alone for all the tumors investigated. These results suggest that mechanism-related *in vitro* assays are useful for read-across prediction of non-genotoxic carcinogenicity of chemical substances.

Presentation: Poster

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Comparative subcutaneous pharmacokinetics and tolerability of standard analgesics in mice and rats

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Rationale: Experimental animal studies often require pain management. Currently, there is a lack of pharmacokinetic (PK) and tolerability profiles in laboratory rodents for various analgesic drugs. The purpose of this study was to provide such data for subcutaneous injection in mice and rats.

Methods: PK plasma profiles of commonly used analgesics were determined by LC-MS/MS in C57BL/6J mice (overall n = 252; 20 mg/kg carprofen, 5 mg/kg meloxicam, 50 mg/kg metamizole, 5 mg/kg butorphanol, 0.1 mg/kg buprenorphine, 25 mg/kg tramadol) and Sprague-Dawley rats (overall n = 159; 5 mg/kg carprofen, 250 mg/kg metamizole, 0.05 mg/kg buprenorphine, 30 mg/kg tramadol) after single s.c. injection. To assess tolerability and side effects, physiological, behavioral and Irwin test parameters were obtained.

Results: In mice, carprofen resulted in a favorable plasma elimination half-life ($t_{½}$) of 8.52 h. PK analysis of all other compounds indicated a $t_{½}$ below 2 h. Opioid analgesics led to mild physiological and behavioral changes. In rats, PK analysis revealed a $t_{½}$ of about 4 h for buprenorphine, of about 2-4 h for tramadol and its major metabolite, of about 4 h for metamizole's major metabolites, and of about 7 h for carprofen. While carprofen was well tolerated, buprenorphine and tramadol altered home-cage behavior and Irwin test parameters, and tramadol injection induced local skin inflammation.

Conclusions: For both species, mainly carprofen appears as a promising candidate for further use in mono- and multimodal analgesia protocols. Due to short half-life and/or tolerability profiles at investigated doses, applicability of the other investigated analgesics for pain management seems limited.

Presentation: Poster

800

New approach methodologies for the hazard assessment of nanocellulose (NC): A tiered approach for the evaluation of the toxicity of NC materials in human intestinal and macrophage models

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Nanocellulose (NC) is an emerging material in the food sector with applications in food packaging, novel food, as well as food additives. The potential hazards of NC are not well characterized and a nano-specific assessment focussing on local toxic effects, and their ability to affect and cross the intestinal epithelium is required. A tiered approach was implemented for the hazard evaluation of a panel of NC samples including nanofibrillated cellulose (NFC), cellulose nanocrystals (CNC) and bacterial nanocellulose (BNC) materials.

In the first tier, a high content analysis-based approach was used to obtain a maximum amount of information on the cellular responses in intestinal and macrophage cell models following exposure to a panel of NFC, CNC and BNC materials. A series of endpoints including cytotoxicity, DNA damage response, oxidative stress, and the pro-inflammatory response was quantified following a 24 h treatment of Caco-2 and THP-1. A selection of NC materials demonstrating significant cytotoxic effects in Tier 1 studies were investigated further in Tier 2 studies where the uptake and crossing of the intestinal barrier was assessed, as well as inflammation and genotoxic potential. Effects of digestion and modification by the human microbiome was also studied. No cytotoxic effects on the panel of endpoints were observed in the intestinal Caco-2 model. However, in differentiated THP-1 cells, while only slight cytotoxic effects were observed, significant increases in pro-inflammatory responses (IL-8 secretion) were observed. Tier 3 testing involved repeated dose toxicity assessment in complex models of the intestinal epithelium.

Presentation: Poster

803

Implementation of clip-on tunnels for enrichment and gentle-handling in all cages of a medium-sized animal facility

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In order to improve the welfare of our breeding and experimental mice, we have introduced plastic tunnels for gentle-handling and as enrichment items in all cages of our animal facility.

Initially, a test was conducted to define the preference of mice between red or transparent tunnels, either placed on the floor or suspended from the upper grid of the cage (IVC model GM500, Tecniplast, Italy). Based on the results of this first test, we opted for transparent clip-on plastic tunnels attached to the grid.

As the tunnel was meant to be used for gentle-handling, all animal caretakers were then trained to gentle-handling techniques in two parts: first, theoretical training to explain the scientific background and basics of handling techniques, followed by a practical session allowing each caretaker to familiarize with the method.

Starting in January 2023, we gradually introduced clip-on tunnels to the currently occupied 6000 cages of our animal facility over a six-week period, bar those from researchers who opposed this approach for various scientific reasons, representing approximately 10% of all cages.

Our first observations indicate a strong usage of this new enrichment by the animals, despite an unanticipated use of the tunnel by certain strains as a latrine.

The general impression is positive among our caretakers. To better evaluate the impact of this change, three months after implementation, a survey was conducted with all animal caretakers.

The detailed results of the tests done, the implementation phase and the survey outcome will be presented and discussed.

Presentation: Poster

804

Development of reliable 3D QSAR models for predicting human thyroid peroxidase inhibitors

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The endocrine system is a complex network of glands and organs that controls and coordinates the physiological and neural systems using hormones. Endocrine disruptors are agents that perturb the endocrine systems and can eventually cause cancerous metabolic equilibration, birth defects, tumors, and other developmental disorders. Thyroid disruptors (TDs) are a subfamily of endocrine disruptors that interfere with thyroid function. Although current test guidelines include measurements of thyroid hormones in circulation, they cannot illustrate the mechanism of action behind thyroid adversity, and screening a greater number of compounds is challenging. Inhibition of enzymes involved in the synthesis of thyroid hormones, such as thyroid peroxidase (TPO) and sodium iodide symporter (NIS), are well-accepted molecular targets. In this study, the human TPO and NIS structures were modelled using homology modelling and validated satisfactorily using Molecular Docking (MD) Simulation. Then, 190 human TPO inhibitors with IC50 were docked with the modelled structure of TPO, and a 3D QSAR model was built using the binding conformation of the molecule with low binding energy as a reference. Machine learning kNN and Random Forest (RF) 3D QSAR universal models were built with R2 values of 0.80 and 0.79, respectively. To validate the developed 3D QSAR models, the TPO inhibition activity of ten small molecules was predicted using the models, and all ten were identified as TPO inhibitors and demonstrated 100% accuracy qualitatively. The experimental results concluded that the models developed were highly reliable in predicting novel molecules as human TPO inhibitors.

Presentation: Poster

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Prediction of the skin sensitization potential of phytochemicals using experimentally validated *in-silico* approach

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In vitro approaches for predicting the skin sensitization potential for chemicals have been widely employed and constant efforts are made to overcome the limitations and challenges associated with them if any. Recent additions of k-DPRA assay, GARD assay and *in silico* predictions with a defined approach to predict hazard as well as potency are described in OECD 497 guidance document.

Phytochemicals which are used as active ingredients or adjuvants in cosmetics and dermal therapeutics, topical applications are frequently observed with skin sensitization and hence it requires caution. Computational approaches like QSAR and read-across appear to be worthwhile in the early phase screening. Since these approaches are limited to structure-based predictions, using system-level approaches in combination would be comprehensive. Hence, in the present study, 603 phytochemicals were screened *in-silico* using OECD QSAR Toolbox. The limitations of the QSAR Toolbox in predicting skin sensitizers were identified and the systems biology-based approach was devised to complement the QSAR-based prediction. Using the devised approach, 41 biomarkers were identified as a determinant of the skin sensitization potential of chemicals, and the molecules attributed to these biomarkers were predicted as skin sensitizers. Further, the relevance of identified biomarkers was studied and validated using an RT-PCR experiment, where THP-1 and HaCaT cells were treated with chemicals. The results justified the role of each approach in making the platform more robust and highlights an opportunity to shift the paradigm of skin sensitization assay with a better understanding of chemical toxicology mechanisms.

Presentation: Poster

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Mode of action of 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors: A new *in vitro* approach to replace *in vivo* measurement of tyrosinemia

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4-hydroxyphenylpyruvate dioxygenase inhibitors (HPPDi) herbicides block the second enzyme of tyrosine catabolism. When HPPD is inhibited, the alternative route for tyrosine catabolism via urinary excretion of HPPD substrate 4 hydroxy-phenylpyruvic acid is via tyrosine aminotransferase (TAT). There are differences in TAT activity between species and this is considered to be the prime factor driving the marked differences in the severity of tyrosinaemia induced by a HPPDi and associated effects (like corneal opacity) observed across experimental species and humans. Previously reported TAT activities in different species showed large variation, were inconsistent, and had methodological uncertainties. In particular, the comparatively high TAT activity in dogs did not match the sporadic observation of corneal opacity in toxicology studies, which posed a reasonable challenge to the hypothesis that species differences were due to differences in TAT activity. To provide clarity, a new and robust *in vitro* method was developed for the simultaneous and systematic measurement of TAT activity in liver cytosolic fractions and/or hepatocytes from a range of mammalian species. The results obtained after full inhibition of HPPD by Nitisinone in primary hepatocyte model showed a very convincing correlation between high TAT activity and low *in vivo* tyrosinaemia-related toxicity. These data support the role of TAT in explaining the species differences in tyrosinaemia-associated toxicity and offer a reliable and reproducible *in vitro* prediction of the *in vivo* response. *In vitro* measurements of TAT activity in hepatocytes turned out to be a reliable method than can be used to replace *in vivo* measurements of tyrosinaemia.

Presentation: Poster

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Development of a method for detecting addictive compounds based on the electrical activity of human iPS cell-derived dopamine neurons

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Addiction is recognized to be triggered by a variety of compounds including commonly used pharmaceuticals, luxury items, and illicit drugs. Thus, it is imperative to develop an evaluation system for side effects of compounds to prevent addiction formation. However, there is currently no validated method to evaluate addiction without resorting to animal testing. In this study, we developed an evaluation system to identify addictive compounds by measuring the electrical activity of human iPSC-derived dopamine neurons using a microelectrode array (MEA). Dopamine neurons (iCells) and astrocytes (iCells) were co-cultured on this MEA. After 35 days of culture, we conducted chronic dosing studies with 10 compounds, and compared the results pre- and post-chronic administration using principal component analysis (PCA). Based on the cumulative dose response before and after chronic administration, all five addictive compounds (nicotine, flunitrazepam, phenobarbital, ethanol, methamphetamine) were identified as addictive compounds. On the other hand, non-addictive compounds (varenicline, amantadine, muscimol, acetaminophen, DMSO) were recognized as non-addictive compounds. This evaluation system also allowed us to discern distinct responses to addictive and non-addictive compounds acting on the same receptor.

These findings suggest that the evaluation system employed in this study serves as an effective screening tool for identifying addictive compounds and that could replace animal experiments.

Presentation: Poster

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From neurotoxicity/DNT screening to hit characterization

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An *in vitro* test battery for DNT (IVB) has been assembled [1]. Some case studies have exemplified how the data may be fed into IATA to support risk assessment. However, no general approach is available on how to understand hits from phenotypic assays on a biological level, and how to link the positive hits to adverse out-

come pathways (AOP). To address this gap of knowledge, we performed an extensive hit characterization study on 7 chemicals found to be positive in a neurite outgrowth assay during a US National Toxicology Program screening campaign [2]. We started with a broad transcriptomics evaluation and several biochemical assays investigating mitochondrial function. A subgroup of compounds seemed to share a toxicity mechanism, related to respiratory chain inhibition. This was further evaluated by metabolomics studies. The unbiased approaches led to the identification of berberine as complex-I inhibitor, which agrees well with the published literature. The metabolomics characterization also provided an explanation, why a compound that may target all respiring cells in the body may have a specific neurotoxic effect *in vivo*. Berberine, and two other mitochondrial toxicants were found to upregulate a neurotoxicity-specific pattern of metabolites. These may act in neurons synergistically with the primary effect of the toxicants on complex-I. Overall the study showed, how a mode of action of unknown compounds may be identified, and how a multi-omics analysis *in vitro* may pinpoint a specific adverse outcome or target organ *in vivo*.

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Presentation: Poster

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An indirect ELISA assay for evaluation of antibody response in rabbits vaccinated with inactivated enterotoxemia vaccine

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Enterotoxemia vaccine is used for providing prophylactic protection against disease which are caused in sheep and goats The pharmacopeia mouse neutralization assay is the standard method for determining the potency of the final vaccine batch by injecting to ten rabbits and measurement of the specific antitoxin concentrations in the rabbit sera by serum neutralization tests in mice that lead to use thousands of mice and usually have lethal endpoints.

The purpose of this study is developing an indirect ELISA assay for identification of *C. perfringens* epsilon and beta antitoxin in rabbit serum that leads to reduction in animal usage. In this method standard serum uses as the positive antigen.

96-well microtiter plates coated with purified *C. perfringens* epsilon and beta toxin overnight at 4°C separately. Blocking was done with bovine serum albumin and then the proper dilutions of sera test was added, in next stage goat antirabbit IgG peroxidase was added. All incubations were at 37°C for one hour and plates washed three times after appending each new reagents, after adding TMB and stop solution and developing the colour at room temperature the absorbance was measured at 450 nm using a spectrophotometer. Antitoxin concentration of the test samples calculated by comparison of absorbance with curve generated using the reference serum.

Mice serve only as indicators for the presence of specific antitoxin concentrations. Since in potency test this kind of ELISA can replaced by mouse neutralization assay but correlations with *in vivo* potency test must evaluate in future research.

Presentation: Poster

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Development and validation of an *in vitro* replacement assay for enterotoxemia vaccine potency test

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Enterotoxemia is a widespread disease of domestic ruminants caused by epsilon toxin of *Clostridium perfringens* type D that results in significant economic losses for the meat and dairy industry. Iran with different climatic provide suitable conditions for sheep breeding (52.2 million heads). Eradication of this disease is virtually impossible; control and prophylaxis are based on good management and systemic vaccination of small ruminant herds with epsilon toxoids.

About 120 million doses of vaccine containing epsilon toxoid are produced annually in Iran. Potency test is one of the most important criteria in quality control of vaccine. As these tests are required for every batch of vaccine produced, this can result in the use of a significant number of animals.

Toxin neutralization tests using cell cultures are available for Clostridial vaccines potency test. In our study, MDCK cell (25,000 cells/ml, 48 hour) was sensitive to epsilon toxin and the cytopathic effect was examined by the MTT staining method. The antitoxin titres of rabbit sera and standard serum (NIBSC, UK) were calculated in mice and cell culture assay (5.9 and 5.5 IU/ml). Good correlation coefficients were obtained between TNT and *in vitro* assay through Pearson's correlation ($r = 0.987$) ($p < 0.01$). This

method does not also require highly purified toxins and monoclonal antibodies that should be used in other *in vitro* methods.

The study results have demonstrated that cell-based assays can be more sensitive and accurate, have high reproducibility and provide huge advantages in terms of saving animal lives.

Presentation: Poster

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Using affordable inanimate tools to enhance hands-on training

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The 3Rs have been adopted internationally as a framework to apply alternatives that improve the welfare of animals used in research studies. Trainers play an impactful role in teaching research personnel how to handle and perform procedures on research animals safely and humanely. Leading by example, it is essential for trainers to incorporate the 3Rs concept into hands-on training. Using inanimate training tools prior to live animal practice is one way to incorporate the 3Rs into hands-on training classes; however, the availability of affordable hands-on training tools is limited. In an effort to improve the use of the 3Rs in hands-on training and make inanimate training tools accessible and affordable to trainers, the author created a variety of inexpensive, hand-crafted inanimate tools to enhance hands-on training of non-surgical and surgical procedures in research rodents and other species. Through years of experience as a trainer, the author identified steps and concepts that were most challenging for trainees to learn and translate to live animal practice. Using this information, tools and exercises were created with the intention of addressing these learning challenges. The approach to hands-on teaching evolved into a trademarked method known as "Translational Training Tools™; the 3Ts Serving the 3Rs (The 3Ts)". The 3Ts method aims to share ideas for affordable and effective means to implement the 3Rs alternatives into hands-on training programs. This poster outlines the features of the 3Ts method and shares ideas for the implementation of the 3Rs for delivering effective, humane hands-on training.

Presentation: Poster

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A microphysiological system for studying human bone biology under simultaneous controlled oxygen tension and mechanical loading

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Throughout life, continuous remodeling is part of human bone biology and depends on the simultaneous fine-tuning of physicochemical parameters such as the oxygen gradient and the variable mechanical load. In order to avoid animal experiments for studies, suitable *in vitro* model systems are needed that allow simultaneous modulation of oxygen tension and mechanical loading as driving factors of bone formation.

Here, we report the development of a novel microphysiological system (MPS) that provides perfusion, independent regulation of the oxygen environment, and precise control of mechanical loading. Our MPS enables quantification and control of applied forces. To better apply the use of the MPS to future studies of the (patho) biology of bone formation, we examined a simplified 3D model of early *de novo* bone formation based on primary human osteoblasts (OBs) seeded into type I collagen scaffolds. OBs were used because they are responsible for bone formation as part of the remodeling process.

Our studies show that with the self-developed MPS we can visualize not only bone matrix formation and mineralization, but also remain cell viability and measure differing metabolism of primary OBs dependent on the respective physicochemical parameters.

In summary, we present an MPS that will allow in the future to mimic (patho)physiological physicochemical changes to better investigate their influence on individual bone biology due to donor-related differences.

Presentation: Poster

824

Sens-ocular model: Cell-based assay to evaluate eye stinging potential of cosmetic formulations

Lara Barroso Brito[1], Carolina Motter Catarino[2], Maria Claudia Passos[1], Artur C. Garcia da Silva[1], Desiree Cigaran Schuck[2], Andrezza Di Pietro Micali Canavez[2] and Marize Campos Valadares[1]

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The TRPV1 receptor, which is known to contribute significantly to pain perception, has recently been identified as a valuable tool for predicting eye-stinging potential in cosmetics. In this study, HEK-293 cells with high TRPV1 expression were utilized to evaluate calcium influx related to receptor activation triggered by chemicals and cosmetic formulations. The cells were exposed to increasing concentrations of 3 non-stinging (carbamide/urea, DMSO, and glycerol) and 4 stinging (ammonium nitrate, citric acid, cocamidopropyl betaine, and sodium lauryl sulfate) reference substances, and TRPV1 activity was assessed by measuring intracellular FURA-2 AM fluorescence signal. To confirm TRPV1 channel activation, capsazepine, a capsaicin antagonist, was employed in addition to using capsaicin as a positive control. The study's results indicate that this novel model can differentiate stinging and non-stinging compounds considering a cut-off value of 60% of Ca^{2+} influx exposed to the lowest evaluated concentration (0.00032%). When applied to the cosmetic formulation, although the presented model exhibited higher sensitivity by classifying as stinging formulations that had previously undergone clinical testing and were deemed non-stinging, the assay could serve as a valuable *in vitro* tool for predicting human eye stinging sensation and it could be used as a tier 1 in an integrated testing strategy. Furthermore, our study has confirmed that the TRPV1 channel is critically involved in the eye-stinging sensation caused by certain cosmetic formulations.

Presentation: Poster

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Assessment of TiO_2 NPs toxicity using a reconstructed human epidermal model

Priscila Laviola Sanches[1,2], Carolina Motter Catarino[3], Bruna Bastos Swinka[3], Andrezza Di Pietro Micali Canavez[3], Desirée Cigaran Schuck[3], Ana Rosa Lopes Ribeiro[4] and José Mauro Granjeiro[5,6]

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Besides ethical issues, using equivalent skin models provides an excellent alternative to animal testing due to its morphological, histological, physiological, and biochemical similarities to native human skin. While some of these models have been validated for assessing chemical formulations' skin irritation and corrosion potential, their suitability for studying the toxicity of formulations containing nanoparticles commonly found in cosmetics remains unknown. Thus, this study aimed to develop a Reconstructed Human Epidermal model (GB-RHE) and evaluate its performance in assessing skin irritation and its potential for assessing the hazards associated with titanium dioxide nanoparticles (TiO_2 NP). In the internal validation of the GB-RHE model, 8 of the 10 reference substances from the OECD TG 439 were correctly classified, resulting in a specificity of 60% and sensitivity of 100%. Subsequently, using the GB-RHE model, the TiO_2 NPs at 10 µg/mL and 100 µg/mL were classified as non-irritant. Although histological analysis (H&E staining) did not show morphological alterations, Transmission Electron Microscopy (TEM) images demonstrated that TiO_2 NPs are internalized and can be found throughout the epidermal layer. This study demonstrates the potential of RHE models for assessing the skin irritation and toxicity potential of nanoparticles and for conducting cellular internalization studies.

Presentation: Poster

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Chemically-induced metabolic disruption: Selection of chemicals for *in vitro* human PPARα, PPARγ, and adipogenesis test method development

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To address metabolic disruption mechanisms and modes of action for chemical hazard assessment, appropriate minimum chemical selection lists have been developed for test method development, refinement, optimisation, and proficiency testing in preparation for (pre-)validation. Mechanisms covered are molecular-level Peroxisome Proliferator Activated Receptor α and γ activation and the tissue/organ-level adverse effect of white adipose tissue adipogenesis.

The lists have been developed with consideration of a number of critical criteria needed for both understanding the strengths and limitations of respective candidate assays, and practical applied aspects as to how candidate test methods may eventually be combined, as part of an Integrated Approach to Testing and Assessment for metabolic disruption. On the basis of the critical selection criteria and considerations for chemicals used in (pre-)validation studies for regulatory purposes, 15 chemicals for PPARα, 17 for PPARγ, and 10 chemicals for adipogenesis were independently peer reviewed and are proposed.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 825489, GOLIATH.

Presentation: Poster

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Vital human tissue as an innovative approach to move towards human-based science without animal research

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The use of human tissue contributed significantly to progress in scientific research. Accessing human tissue for scientific research is however difficult. Fresh, vital tissue is being used more frequently and considered to be of high translational value, but several barriers and constraints exist with respect to feasibility and (regulatory) acceptance. Material can be used as long as ethical and legal regulations, such as the written consent of patients, are followed in a transparent manner. However, several challenges exist. We aim to get insight in the drivers and barriers to use vital human tissue, and to propose concrete actions to overcome the challenges.

Overall, this project aims to contribute to the transition from animal-based to human-based safety and efficacy evaluation in the food and nutrition sector. Regulators, NGO, academia, and industry stress the need to move towards human-based science. Data and information generated with vital human tissue will give insight in the predictive value for the human situation, in comparison to those obtained from cell lines or animal tests. However, in most (European) countries there is no transparent infrastructure to have access to human tissue for scientific research.

A survey was initiated to obtain information on the stakeholder needs and constraints. There are several logistic, quality, safety, ethical and cultural issues to overcome. Results of the survey and the learnings of the Dutch feasibility study will contribute to a guidance document, including a roadmap for an infrastructure in European countries to make vital human tissue available for scientific research.

Presentation: Poster

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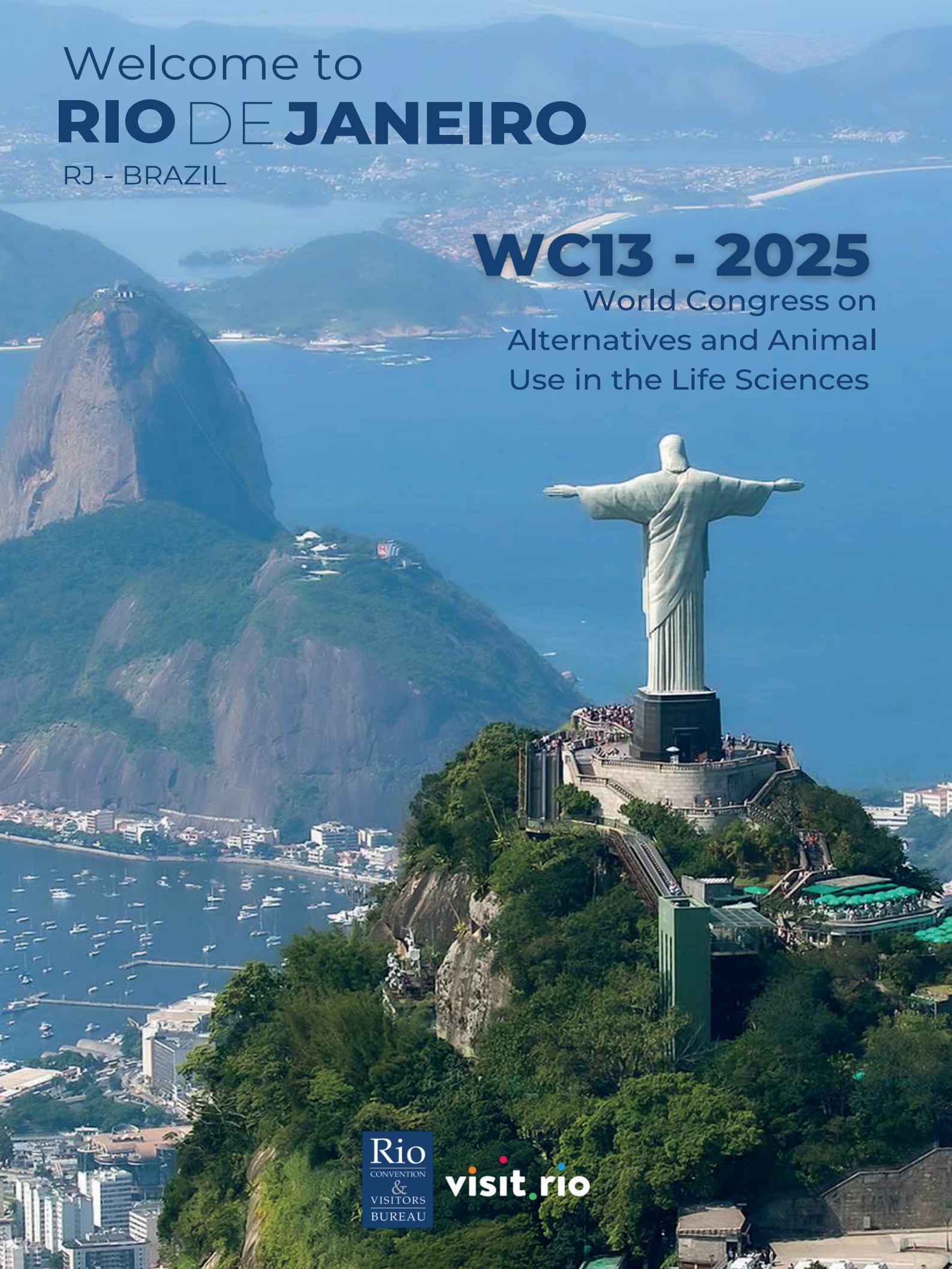
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