



# Abstracts of the 11<sup>th</sup> World Congress, Maastricht, 2021

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# ALTEX Proceedings

Jos Kleinjans  
and  
Pascalie Van Loo  
**Welcome**



Theme I  
**Safety**

Theme II  
**Innovative Technologies**

Theme III  
**Ethics, Welfare and  
Regulation**

Theme IV  
**Disease**



# WC11 VIRTUAL CONGRESS

## Welcome

Dear colleagues,

Welcome to 11th edition of the World Congress on Alternatives and Animal Use in the Life Sciences! Originally, in a pre-COVID19 era (can you still remember?), foreseen to be held in the city of Maastricht in The Netherlands, but – since the virus is still raging on across the world – now presented to you via the World Wide Web. This, of course, is rather unfortunate because we cannot offer you the great hospitality our city is famous for, and having spontaneous conversations digitally is not that obvious either. But we, as the Local Organizing Committee, took these potential downsides as a challenge to bring to you an innovative platform which should go beyond a generic series of online PowerPoint presentations. I believe we managed to develop great graphics to create a virtual, but realistic congress environment. We have added a few features (such as 3 talk shows) where lively discussions can be initiated, and due to the advances of IT technology, allow interactions across the globe. We hope that you will appreciate it.

As an overarching theme for designing the scientific program we have chosen: “3Rs in transition: from development to application”. This has been inspired by the observation that in the last decade tremendous progress has been made in a wide range to technologies (stem cells, organ-on-a-chip, genomics, micro-engineering, ...) all supportive for realizing non-animal test models of the highest grade, and boosting scientific research in the 3Rs, and in particular Replacement, to a yet unmet level, whilst acceptance of such new generation models by the various application domains is still quite low. We aim to explore this seeming discrepancy, not only in the field of chemical safety testing, but also in vaccine development, and certainly also in creating relevant human disease models. We hope that you will find this inspiring for your own efforts in the 3Rs.

Again, a warm welcome to the virtual congress and we hope you enjoy, get inspired and connected!

**Jos Kleinjans,**

Chair - Local Organizing Committee WC11





**“Wherever possible,  
specialists should not be segregated in  
separate laboratories. The aim should  
rather be to assemble as many different  
kinds as possible under one roof.”**

William Russell and Rex Burch,  
founding fathers of the 3Rs,  
in *The Principles of Humane  
Experimental Technique*, 1959.

In line with this philosophy, the World Congress on Alternatives and Animal Use in the Life Sciences has been a triannual event that brings together specialists in the field of the 3Rs and closely related subjects. Despite the challenges we face in these times of COVID19, where videoconferencing is the norm and interacting on a personal level is reduced to a minimum, we have created congress surroundings that stimulate the exchange of scientific ideas and inspire you to connect to colleagues all over the globe.

In over 100 symposia, workshops and key note lectures, distinguished experts as well as young scientists share with us the fruit of their recent work on innovative non-animal methods, good research practice, harmonization, education, transition towards animal free research, ethics, etc.. We have created networking areas where you can meet old friends and promising new contacts to exchange exciting ideas and forge bonds for future cooperation.

If you are an early career scientist, we want you to feel particularly welcome! We have collaborated with YOU-WC11 to organize several interactive sessions and events that promote dialogue amongst yourselves and with experienced peers. You are the future, we invite you to learn, share and challenge current views!

Organizing a world conference is no chick feed, but many hands make light work. We have much enjoyed putting together the scientific program and are very thankful for the help of the members of the international scientific committee, the local organizing committee, the many session organizers and of course our sponsors.

We hope that this virtual congress will bring you an experience you never to forget. Enjoy!

**Pascalie Van Loo**

Chair-International Scientific Committee



**Dear WC11 participants,**

ALTEX Proceedings is honored to publish the Abstract Book of the 11<sup>th</sup> World Congress on Alternatives and Animal Use in the Life Sciences in Maastricht, The Netherlands.

Owing to the ongoing global pandemic of coronavirus disease 2019 (COVID-19), the Congress will be held as a fully virtual congress for the first time, one year after the time it originally was going to take place. Although we will not have the chance to visit Maastricht on this occasion, we look forward to sharing this exciting experience with you and hope all participants will make the best of the new opportunities the virtual congress offers. To allow participation from the different time zones of the world, the congress will be spread over nine days with a free weekend in the middle. Please consult the WC11 Magazine and the congress website <https://www.wc11maastricht.org/> for information on the program.

This Abstract Book contains short summaries of almost 800 abstracts that were accepted for oral or poster presentations. They are sorted by presentation type and submission ID number. The abstracts address the four Congress Themes: Safety; Innovative Technol-

ogies; Ethics, Welfare and Regulation; and Disease. They represent the work of contributing authors from a total of 42 countries from six continents.

We thank Robbin Grouwels and Ramon Litjens from Klinkhamer Group for his cooperation in producing the Abstract Book. We are grateful to the Doerenkamp-Zbinden Foundation, Switzerland for again generously funding the production of the Abstract Book.

We wish all participants of WC11 that the congress will inform, connect, and inspire you to drive forward the exciting field of alternatives to animal experimentation.

We can now already look forward to meeting again in person at WC12, which will be held in Niagara Falls, Canada in August 2023.

With best wishes,

Sonja von Aulock  
Editor in chief, ALTEX & ALTEX Proceedings



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# Oral Presentations

1

## Animal free multiclonal antibody generation as a replacement for polyclonal antibodies

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While phage display, the premier animal free antibody generation method, is well established for the generation of therapeutics, most antibodies for research and diagnostics are still made using animals. This presentation reviews the achievements and prospects of recombinant *in vitro* antibody generation, demonstrating how animal derived antibodies could be complemented or replaced in a large number of typical current research applications. Examples of further advantages of the *in vitro* antibody generation will be presented in respect of predesigned features and sequence defined quality. These will include how polyclonal animal-derived antibodies may be replaced by recombinant multiclonal antibodies in various applications ranging from the typical secondary antibodies used in research to horse sera used for therapy by passive vaccination.

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**Presentation:** Oral

2

## Incorporation of a metabolic component into *in vitro* Tox21 high throughput screening assays

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The US Tox21 Program has utilized a quantitative high throughput screening (qHTS) approach to profile thousands of environmental chemicals using a battery of *in vitro* cell-based assays. An important limitation of these assays, particularly those that measure events associated with DNA damage and repair (i.e., genotoxicity), is the absence of a xenobiotic metabolism capability. The absence of a method to provide for metabolic transformation of parent compounds may potentially lead to mischaracterization of exposure hazard if the parent compound is detoxified *in vivo*, rendering it less hazardous, or alternatively, bioactivated *in vivo* to a hazardous metabolite. To overcome this limitation, we investigated methods to incorporate a metabolic component (e.g., human liver microsomes) into existing Tox21 assays. This presentation will provide an overview of the Tox21 efforts to incorporate metabolism into *in vitro* HTS assays, and will be followed by presentation of a case study of a screening approach to identify acetylcholinesterase inhibitors whose activity is dependent on metabolic activation.

**Presentation:** Oral

3

## The need to prioritize “Replacement” in Alzheimer’s disease research

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Animal models of Alzheimer’s disease (AD) have been extensively utilized in the last decades to elucidate the pathophysiological mechanisms of the disease and to test novel therapeutic drugs. However, basic/fundamental and pre-clinical research successes have not translated into effective therapeutic treatments for AD patients. One of the possible reasons behind this translational failure may be



the over-reliance on animal models for AD, which have been shown useful to recapitulate some AD-associated features, such as amyloidosis and tauopathy, but have failed to deliver effective treatments for AD patients. On the other hand, the use and the implementation of human-based methods, non-invasive neuroimaging technologies, and large scale epidemiological data set repositories, may contribute to the development of new preventive and treatment strategies.

Here we discuss the need to prioritize replacement, over refinement and reduction, in AD research, and show how we can mitigate this translational gap by employing human-based methods to elucidate disease processes occurring at multiple levels of biological complexity. A paradigm shift towards human-based research, accounting for a multi-dimensional and multi-disciplinary approach is highly needed to tackle the ever-increasing prevalence of AD.

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**Presentation:** Oral

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## The transparency agreement in Spain: An example of success

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The Transparency Agreement in Spain was launched in 2016 by the Spanish Confederation of Scientific Organizations (COSCE) with the help of the European Animal Research Association (EARA) and the Spanish Association for Laboratory Animal Science (SECAL). The Agreement has four commitments following the example of the Concordate on Openness on Animal Research in the UK. The Agreement reached more than 140 signatories including research institutions, professional organizations, and some patient groups. Three Annual Reports have been published, which describe the di-

verse activities performed by signatories, and the fourth is in progress. The reports show first the awakening of the research community in terms of openness and second demonstrate the consolidation of this way of communication with the public. The Agreement has marked a before and an after on openness. For example, currently 100% of signatories have a public statement on the use of animals on the website. Other activities include visits to animal facilities, communication with the press, outreach activities, etc. Negative reactions to the signing of the Agreement have not been reported. The cooperation between the national scientific (user) community and laboratory animal professionals, and the international specialists on communication has proved to be the most important reason for success, especially in an environment where there is not a consolidated national organization working on communicating animal research. Practicalities of how to promote successfully a national openness/transparency agreement will be discussed and suggestions given regarding different national situations.

**Presentation:** Oral

5

## The need to address human relevance and measure impact and innovation of biomedical research

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Animal models have been traditionally used in biomedical research to recapitulate human disease features and develop new drugs, as they are generally purported to resemble some of the major hallmarks of human diseases. However, these animals do not develop the disease as it occurs in humans, and their use has not paved the way to the development of drugs effective in human patients. Indeed, despite conspicuous research and economical endeavors, the clinical failure rate in drug development still remains very high, with an overall likelihood of approval from Phase I of about 9.6%. On the other hand, the expanding toolbox of non-animal methods, accounting for, e.g., induced pluripotent stem cells derived from patients, next-generation sequencing, omics and integrated computer modelling can be used to study human diseases in human-based settings, identify new potential druggable targets, and evaluate treatment effects. The rise of new technological tools and models in life science, and the increasing need for multidisciplinary approaches, have encouraged many research initiatives and the launch of new EU calls for proposals. Research proposals based on the use of both animal and/or non-animal approaches have been extensively funded at Euro-



pean level. Nowadays, it is becoming pivotal to define and apply indicators suitable to measure social impact of research funding strategies, monitor contribution to innovation, retrospectively assess public health trends, and readdress funding strategies when needed. Here we discuss such issues, describing a list of indicators to measure impact and innovation of biomedical research.

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**Presentation:** Oral

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## How to improve training in an EU organoid training network

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organovir (Organoids for Virology) is a novel European consortium consisting of 15 partners from 7 European countries. Two principal investigators from the Amsterdam UMC, location AMC, Katja Wolthers (Clinical Virology) and Dasja Pajkrt (Pediatric Infectious Diseases) coordinate this project in which 15 Early Stage Researchers (ESRs) follow an innovative training programme. organovir aims to train young researchers in a consortium of public and private experts that will enable them to lead innovation in the field of organoids for virus research.

The training programme consists of the following parts: network-wide international, intersectoral and multidisciplinary training; mentorship from experts from disciplines and sectors other than the primary ESR project. A specialized pre-MBA programme is designed specifically for organovir that will enable the ESRs to go beyond the lab, from scientists to entrepreneurs. All ESRs will develop a personal developmental plan (PDP) (named Beyond

dU) for their self-development and career planning that will focus on the following aspects: own ambition and opportunities in career development, gender issues, work-life balance, strengths and weaknesses, opportunities and threats. It is a state-of-the-art blended learning programme for personal growth; Delivering the next generation of 21<sup>st</sup> century leaders; well-rounded, highly skilled and capable individuals, who are a human at work and a role model for being confident and resilient leaders of the future.

**Presentation:** Oral

7

## Available and emerging non-animal models for human respiratory tract diseases

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This project, funded by the European Commission's Joint Research Centre (JRC), aimed to provide a snapshot of the non-animal models currently used for research into human respiratory tract diseases, and to explore where models were emerging or new models were required. Over 21,000 abstracts were scanned for relevant non-animal methods of respiratory disease, with a total of 284 publications identified as being promising candidate methods according to a set of inclusion/exclusion criteria. *In vitro* cell and tissue cultures (including organ chips and organoids), human *ex vivo*, and *in silico* approaches were the main methodologies considered.

This review shows that, while simple models are still prominent and have their uses, research focus has, in the past 5 years, been shifting towards increasingly sophisticated bioengineering approaches that recapitulate lung development, tissue architecture and physiological functions *in vitro*. Such approaches hold the promise of more human-relevant disease models that can be used to elucidate mechanism of disease and aid in the development of new therapies. However, we also recognized several key areas that need to be addressed to promote the uptake, development, and standardization of non-animal models for respiratory disease research. These include the creation of SOP for model generation and testing; promoting the use of more physiologically relevant substrates; enabling better access to human tissue; and promoting more effective cross-talk between oncology and respiratory diseases.

These methods have been collated into a JRC Data Catalogue of biomedical disease models that will form a key knowledge source for researchers, educators and national ethics and funding authorities. The availability of a centralized source of reviewed methods will go some way to extending the requirements of EU Directive 2010/63/EU on the protection of animals used for scientific purposes to biomedical science.

**Presentation:** Oral



8

## Talking about harms

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In recent years, a number of countries have launched initiatives to help improve openness and transparency on animal research. Usually, the stated aim is to enable people to “access a comprehensive body of information about animal research so that they can debate the issues from a position of knowledge and understanding of the facts so that they can make up their own minds about animal research”. For any “openness” initiative to be genuinely credible, this information should include meaningful and honest details regarding what animals actually experience.

Signatories to such initiatives have made a range of commitments, including “When we communicate about the use of animals in research, we should provide accurate descriptions of the benefits, harms and limitations of such research, be realistic about the potential outputs of such research, and be open about its impact on animal welfare and the ethical considerations involved”. However, in practice it is clear that “providing a balanced communication of the harms as well as the benefits involved in animal research remains a significant challenge for organisations” and this commitment is currently often the least well met.

Many in the research sector might find talking about the harms experienced by animals the most challenging aspect of meeting their “openness” commitments, and of public engagement in general. However, a number of individuals and organisations are making progress, using a variety of different media to acknowledge, describe and show what animals used in research can experience when they are used in procedures. Significant improvements are still needed in order to achieve true openness and enable members of the public to reach informed opinions.

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**Presentation:** Oral

9

## An adverse outcome pathway (AOP)-informed integrated approach to testing and assessment (IATA) as a tool to conduct a developmental neurotoxicity (DNT) hazard characterization

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New approaches in toxicology including the AOP and IATA concepts, *in vitro* assays based on human cells and *in silico* modeling used in an integrated manner, may pave the way to a more efficient and predictive assessment of DNT, solving various regulatory challenges (Bal-Price et al., 2018).

Towards this goal, the EFSA PPR Panel has developed an AOP-informed IATA as a tool to conduct DNT hazard characterization for IATA. Deltamethrin and flufenacet were selected for IATA case studies. A systematic literature review was conducted for human epidemiological studies, animal data (including *in-vivo* regulatory studies), *in-vitro* and zebrafish data.

The AOP framework was applied to integrate information from these lines of evidence, and the DNT *in vitro* data from battery of assays (DNT-IVB) anchored to key neurodevelopmental processes. Uncertainty analyses were performed for each type of evidence to support conclusions on the hazard identification/characterization, and to express the uncertainty in a probabilistic way. This stepwise approach resulted in the development of an evidence-based AOP network for deltamethrin with a probabilistic quantitative estimation of the weight-of-evidence (WoE) using a Bayesian network approach.

This AOP network consisted of two MIEs leading to altered behavioral function (the adverse outcome). The case studies showed the applicability of the DNT-IVB for hazard characterization and illustrated the usefulness of a developed AOP network and probabilistic quantification of the WoE for regulatory decision making. Mechanistic understanding facilitated a human-relevant adverse outcomes interpretation, supporting the contextualization of these studies in the risk assessment process.



Based on this information and experimentally generated new *in vitro* data, an OECD Guidance Document on *in vitro* DNT test methods within the context of an IATA is under development (Sachana et al., 2019) in collaboration with EFSA and DNT experts from the OECD member countries and should be finalized before the end 2021.

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**Presentation:** Oral

10

## Use of human cell lines with different bioactivation capacities to determine the genotoxic mechanism of action

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The development of *in vitro* genotoxicity assays as an alternative to animal experimentation is of growing interest. However, extrapolation of toxicity data between *in vitro* systems and an *in vivo* situation is hampered by differences in the biotransformation of chemicals. In this context, attention must be paid to cell models because they are contributory factors to the final result. Using different radioactive chemicals (e.g., bisphenols, polycyclic aromatic hydrocarbons), we determined the precise biotransformation capacities of different human cells lines used in genotoxicity studies (e.g., HepG2, HepaRG, ACHN, LS-174T). In parallel, we have developed and validated a new genotoxicity assay based on histones H2AX and H3 quantification. This novel method permit to discriminate efficiently aneugens, clastogens and cytotoxic compounds in all cellular models. This assay coupled with the used of human cell lines with different bioactivation capacities permit to differentiated proficiently direct genotoxins from bioactivated ones. This proposed new genotoxicity screening strategy may provide more physiologically relevant data for chemical risk assessment.

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**Presentation:** Oral

11

## Modernizing the NTP's Carcinogenicity Testing Program

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The National Toxicology Program Health Effects Innovation (HEI) initiative on carcinogenicity testing in the 21<sup>st</sup> Century has been launched in 2019 to develop modern, human-relevant, efficient approaches to test for chemical carcinogenicity. This talk will discuss the motivation behind an evolving paradigm for cancer assessment, including evidence for environmental chemical contributions and a desire for tools to screen and prioritize substances, to make actionable predictions, and to provide mechanistic insight. Topics covered will include the translational pipeline concept, the evolution of the testing program, the integration of Tox21 approaches, and designing strategies to fit regulatory needs and answer critical human health questions.

**Presentation:** Oral

12

## Animal welfare assessment of genetically altered Göttingen Minipigs

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Göttingen Minipigs have for decades been used in biomedical research as sharing many anatomical, physiological, and pathophysiological similarities to humans, and as such playing an important role as large animal models in translational studies. In recent years, the number of genetically altered Göttingen Minipigs has increased as advanced genetic techniques simplify the generation of animals with precisely tailored modifications designed to replicate lesions responsible for human disease. As such, genetically altered Göttingen Minipigs are valuable large animal disease models and in addition considered promising donors for xenotransplantation.

To ensure compliance to the 3Rs and ensuring high animal welfare standards, it is crucial to perform a baseline assessment of the



welfare of all genetically altered Göttingen Minipigs models; both during the time of creation, but also followed by the time of maintenance of the specified genetically altered model. Such a welfare assessment should be performed by experienced and knowledgeable staff, and should include animals of representative age groups soon after birth, around weaning and again around sexual maturity and include both males and females and data from a minimum of two breeding cycles. Very importantly, all comparisons should be made with similar non-genetically altered animals representing the same background strain. The observations should be performed in a structured way and include appearance, like body condition, coat and skin condition; body functions, like food and water intake; environment, like defecation and urination; behavior, like social interaction, posture and mobility; procedure-specific indicators, based on the individual project and potential adverse effects; plus finally free observations including all unexpected negative welfare impacts. In addition, an individual welfare assessment should be performed in neonatal animals based on skin color and appearance, activity level, interaction with the sow, suckling behavior and litter related data, like gestation length, litter size and development and growth of piglets.

**Presentation:** Oral

13

## Accelerating regulatory use of alternatives in DART testing: How to build confidence

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The greatest revolution that will define the next era of toxicity testing is the shift from black-box driven standard animal experiments towards tailor-made mechanistic approaches by intelligent and pragmatic testing using computational, molecular, and *in vitro* tools. This change will apply for pharmaceuticals, agrochemicals and industrial chemicals, and is for pharmaceuticals in line with the recent update of the ICH S5 guideline to better reflect the revolution in scientific, technological and regulatory knowledge. A more mechanism-based approach to toxicity testing has the benefit of “opening the block-box” to reduce the need for animal testing or eliminate it altogether. For example, the Dutch government officially announced in 2016 their resolution to become a world leader in animal-free innovations by 2025. Moreover, in 2019 the US EPA announced a reduction of requests for, and funding of, mammal studies by 30% by 2025 and elimination of all mammal study requests and funding by 2035. This can only happen with interdisciplinary efforts that bring together regulatory research, fundamental scientific research, applied and translational research, and education/training. As shown during the first four presentations of this symposium, scientifically we have made huge progress. However, most stakeholders still lack con-

fidence to change our standard methods (i.e., animal testing as the gold standard). A critical need exists, therefore, to build confidence in the new approach methodologies (NAMs) as a reliable and human-relevant paradigm for toxicity testing. Communication strategies and educational programs must be built to then accelerate regulatory use of alternatives in DART testing.

**Presentation:** Oral

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## Openness in the UK since the Concordat

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The UK published the world’s first Concordat on Openness on Animal Research in May 2014. In the six years since its publication the UK has seen a significant increase in the amount of information available to the public on why and how animals are used in research. Numerous photographs, videos and virtual tours can now be found online, as can details of the numbers and species of animals used in experiments. Signatories to the Concordat are encouraged to be realistic in their communications, acknowledging the harms to the animals and the limitations of animal research as well as its benefits and successes. Public opinion surveys show that the percentage of the public in Great Britain that consider animal research establishments to be secretive is slowly decreasing, while the percentage that consider themselves to be “well informed” about the use of animals in research is slowly increasing. Understanding Animal Research continues to support Concordat signatory organisations with communications training, information-sharing workshops, newsletters and an annual Openness Awards ceremony. In 2019 UAR introduced a new “Leaders in Openness” category to recognise those signatories that have demonstrated excellent practice in engaging with the public on this issue.

**Presentation:** Oral

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## The key characteristics of carcinogens

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The key characteristics (KCs) of carcinogens are a collection of characteristics of chemicals known to cause cancer in lab animals and humans. The key characteristics are used to identify available mechanistic evidence that an agent can cause cancer in humans. Together with evidence on cancer in experimen-



tal animals and epidemiological evidence on cancer in humans, the three evidence streams are integrated in an overall evaluation. This approach has been successfully used to classify the hazard of agents under consideration by the International Agency for Research on Cancer (IARC). Despite an absence of a full understanding of how the KCs of an agent may lead to carcinogenicity, the KC approach has been used to transparently and systematically identify mechanistic information, and integrate diverse data streams in an overall classification of carcinogenic hazard.

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**Presentation:** Oral

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## Application of the key characteristics in carcinogen hazard identification

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Smith et al. (2016) identified 10 key characteristics, one or more of which are commonly exhibited by established human carcinogens. The key characteristics of carcinogens reflect the chemical and biological properties of cancer-causing agents and are distinct from the hallmarks of cancer, which are the properties of tumors. The key characteristics have been applied in evaluating mechanistic data for more than 50 diverse agents in recent IARC Monograph meetings. Because the key characteristics are based on empirical observations of characteristics associated with known carcinogens, they provided an agnostic and unbiased survey of the mechanistic literature. This improved uniformity across evaluations, revealing strengths as well as gaps in evidence, and highlighting mechanistic similarities and differences in the agents considered. Based on this experience, the 2019 update to the IARC Monographs Preamble adopted an ap-

proach based on the key characteristics to identify and review mechanistic evidence relevant to identifying human carcinogens. This is reflected in harmonized approaches to evidence evaluation across scientific disciplines as well as in a single-step integration of mechanistic, animal bioassay, and human cancer evidence streams. These are important advancements for identifying the causes of human cancer, the first step in cancer prevention.

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**Presentation:** Oral

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## 3Rs opportunities in preclinical safety testing: A CRO perspective

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The 3Rs concept has been widely adopted and is now included as recommendations from regulatory agencies in global drug development guidelines, e.g., recent ICH guidelines. This presentation focusses on 3Rs opportunities when using “large” animals (dog, minipig and non-human primate (NHP)) in preclinical safety assessment. From a Contract Research Organisation (CRO) perspective, there are infrequent opportunities to replace animals in safety studies due to the paucity of validated non-animal approaches that are accepted by regulatory authorities. In contrast, there are ample opportunities for reducing animal numbers, and for refining the design and conduct of toxicity studies, and significant progress has been (and continues to be) achieved. Selected examples include enrichment standards, performance behavior-based acclimation, improvements in animal housing for urine/feces collection, > 90% reduction and enhanced welfare in minipig use for DMPK evaluations, social housing of dogs and NHPs for ADME studies and cardiovascular telemetry as-



assessments within toxicology studies, home cage-based cognitive tests for NHPs, and an approx. 50% reduction in the number of NHPs required for biopharmaceutical drug development. In addition, in our experience, social housing concepts yield comparable data across different sites, e.g., for pregnancy success and infant growth in developmental toxicity studies. Notably, the development of highly targeted biopharmaceuticals/oligonucleotides and gene therapy have led to an increased demand for the NHP model – therefore, refinement and reduction continue to be key objectives when using these models.

**Presentation:** Oral

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## The current translational gap: Problems and solutions

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Decades of intense research using a variety of mouse Alzheimer models has not yet translated into significant benefits for the patients. Mice do not develop Alzheimer the way humans do despite being genetically modified with the genes that are involved familial (early-onset) Alzheimer's disease (fAD) and/or over-expressing proteins that are crucial for pathogenesis: amyloid- $\beta$  and tau. This disconnection from the human condition is not sufficiently considered.

While clinically like fAD (1%), the onset of sporadic (late-onset) Alzheimer disease (sAD) (99%) has age as major risk factor with prevalence creasing in individuals over 65 years of age. Environmental factors and lifestyle may also play an important role. There has been disproportionately little interest in these external factors.

Maintaining good quality of life when struck by a disease leading to irreversible brain damage requires a therapy targeting processes that downstream early clinical symptoms, and diagnostic tools that identifies individuals at risk at an early preclinical stage. Yet, the affected early mechanisms and processes remain therefore elusive.

To acquire a better understanding about the early pathology, existing mechanistic data on environmental neurotoxic chemicals affecting cellular mechanisms that are frequently associated with AD pathogenesis are structured according to the adverse outcome pathway (AOP) (OECD, 2016) a publicly available program permitting tailored application for a range of purposes. A biologically plausible mechanistic scheme of sequential events associated with early pathogenesis of sAD was constructed. The proposed molecular initiation events (MEI) may trigger a sequence of key events, causally linked to each other via key event relationships, causing neuronal cell damage and eventually deficits in memory and learning process, and sAD.

In this perspective, the proposed serial events may be supportive for the development of predictive biomarkers which are urgently needed for filling in the gap at early phase diagnosis of AD.

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**Presentation:** Oral

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## How to better highlight your research by using the right keywords in titles and abstracts

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With around 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, it is now more than ever vitally important to make your work stand out. Often the first openly-available information about your latest publication will be the title and the abstract, so careful consideration need to be given to these elements of research to ensure that they create the right impact, attract the right readership and allow your work to progress through further citation, visibility and continued/increased funding!

However, creating a title, abstract and keywords can often be neglected and may only be considered at the last minute before publication, or written according to the journal guidelines, with little thought for how other researchers will find and appreciate the research. This session will look at how to ensure that your title and abstract are eye-catching and appropriate- allowing your audience to understand the importance of your research whilst encouraging them to access and read the full paper. We will look at effective use of relevant (Google Scholar-friendly) keywords to ensure that your article will be easily retrieved, consider the key characteristics of a title and examine how to ensure the abstract addresses the key objectives and conclusions of the paper.

**Presentation:** Oral



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## Overview of new approaches in biomedical research – The BioMed21 collaboration

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Drug development is broken. The current animal-based research methods used through the drug development pipeline can take up to ten years and millions of pounds to develop a single pharmaceutical. Even then, there is a more than 95% failure rate in human trials- the majority for reasons of unexplained toxicity, safety or lack of efficacy. This is a highly inefficient model that has seen the number of new drugs reaching the market plummet in recent years and leaves us at risk of a drugs drought unless we revise the testing paradigm.

The biomedical research for the 21<sup>st</sup> century (BioMed21) collaboration aims to accelerate the paradigm shift. BioMed21 was borne out of a 2015 review publication authored by a diverse, international group of stakeholders representing animal protection, research funding, academic, regulatory, corporate, and other communities. This need for change was catalyzed by the scientifically valid, human-relevant principles of the Tox21 initiative that envisioned a toxicity testing system using computational biology and other *in vitro*-based assay systems.

This presentation will discuss the translational limitations of the current paradigm in biomedical research and drug discovery, illustrating that the need for change lies in the development and application of more human relevant, human predictive systems. It will cover the ways in which the BioMed21 collaboration are intervening to enable this paradigm shift and introduce case studies of different human conditions as examples of where and how the modern human-relevant tools may be used to offer more insight into disease progression and pathogenesis than continued reliance on animal models could ever do.

**Presentation:** Oral

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## Potential refinement of animal models of neuropathic and inflammatory pain

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The use of animal models in research for understanding mechanism and identify potential treatment targets of pain is necessary at present. This is indeed problematic from an ethical and animal

perspective, as these models are generally classified as severe. Therefore, there is a need for thorough research with the focus of refining these models to every extent possible. In our research group, we have for the past years engaged in studying possible refinement strategies for rat and mouse models of neuropathic and inflammatory pain. The focus of these studies has been on developing refinement through analgesic treatment in connection with surgical induction or during model development. Furthermore, focus has been on identifying more sensitive parameters, in order to recognize impaired welfare at an earlier stage. The present talk will present an overview of the results and conclusions of these and other related studies. It can be concluded that analgesic treatment can be applied to these models without compromising the validity of the model in many cases. In addition, it can be concluded that there is still a great need for identifying novel welfare parameters that are sensitive enough to detect early changes in welfare impairment, which is necessary for developing future refinement strategies.

**Presentation:** Oral

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## Re-homing rodents – A university perspective

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Although re-homing former laboratory animals, such as cats and dogs, has been practiced in some laboratories on a voluntary basis for decades and some national recommendations for the placement of laboratory animals exist, efforts to re-home laboratory animals have been further stimulated by the publication of Directive 2010/63/EU in the EU Member States. For Switzerland, there is currently no national recommendation on the re-homing of laboratory animals.

While there are some well-known re-homing programs for cats and dogs, the re-homing of smaller laboratory animals such as rodents, on the other hand, is less well known. In autumn 2018, a collaboration between the Swiss Animal Protection (SAP/STS) and the University of Zurich resulted in a re-homing project with the aim of giving rodents and rabbits from animal experiments a new life in private homes. For experimental and legal reasons not all laboratory animals can be re-homed after the experiments. However, until now more than 200 rabbits, rats and mice have already been successfully re-homed. The re-homing project receives great support from the experimental animal husbandries and the research groups involved.

In this talk, I will present the prerequisites, challenges and potential of the UZH rodent re-homing program.

**Presentation:** Oral



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## NORECOPA: A hub of international 3R resources

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The incorporation of the 3Rs in national legislation in many countries, the recent emergence of many new 3R Centres and Societies, and current concerns about the validity, reproducibility and translatability of animal research, have all created a need for easy access to information about 3R institutions and quality-controlled resources which can advance the 3Rs.

Norecopa (<https://norecopa.no>) has proved to be in an ideal position to perform this task, with its experience in database construction, 3R resource dissemination and networking in the international research animal community since the early 1990's. Funding from external sources made it possible to construct a state-of-the-art website, launched in 2016, which merged many individual resources into one, searchable, database, currently with over 9,000 pages. The metrics for this website, with over 300,000 page views in 2020, suggest that the service is valued worldwide.

This presentation will describe some of the latest initiatives conducted by Norecopa and its collaborators to collate and disseminate information on best practice and the 3Rs. These include:

An interactive map of global 3R Centres, with links to each Centre;

- A Refinement Wiki, where registered users can publish details of refinements of animal care and use;
- The website of the International Culture of Care Network;
- The PREPARE guidelines for planning animal experiments, with a checklist which is now available in 25 languages;
- Integration of 3R resources produced by the EU Commission;
- A compilation of severity classification systems, making it easier to compare and decide upon the level of procedure severity when planning or reporting animal studies;
- Detailed newsletters issued 7-8 times a year, with information about the latest developments within the 3Rs worldwide.

**Presentation:** Oral

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## Is wildlife research “second-rate science”? What can lab animal and field scientists learn from one another?

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There are fundamental differences between research carried out on companion, farm and lab animals and that performed on wildlife, where the period of interaction is often very short, albeit intense. We instinctively acknowledge our responsibilities for captive animals from birth until death. Our responsibility for the welfare of animals used in wildlife research, on the other hand, is generally considered to be much more limited: the natural laws of predation and disease run their course, both before and after our interventions. However, it is in scientists' own interests to ensure that interventions such as tagging and collaring following this short period of captivity cause negligible effects on animal welfare, to avoid invalidating the study data.

To a wildlife researcher, the 3R principles were very clearly developed in another scientific era and for use on traditional laboratory animals. The question is whether the comprehensive ethical and practical guidance which we have developed, not least after the advent of EU Directive 2010/63, for use on captive animals can be transferred without further adjustments to wildlife.

This presentation will examine the extent to which wildlife researchers have implemented the principles embodied in the 3Rs, and challenges that arise in complying with legislation such as Directive 2010/63. The aim of the presentation is to discuss how we can contribute to better mutual understanding between scientists, enhanced animal welfare and more valid research. It will also give an overview of the guidance which has been identified by international consensus meetings on wildlife research arranged by Norecopa and others (<https://norecopa.no/meetings>).

**Presentation:** Oral

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## Artificial intelligence for drug safety and biomarker development

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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 machine learning approaches with a specific emphasis on learning from the existing data/information to predict future outcomes. The concept of AI was introduced back in 1950s



and its critical role in a broad range of application has yet been realized. In this presentation, the basic concept and methodologies of AI will first be introduced. The discussion will then be directed to apply AI for drug safety and biomarker development. With examples from prediction of drug induced liver injury and gene expression based predicative models, the lessons-learned and guiding principle will be discussed with respect implementing best practice of AI in risk assessment and clinical application. The rise of AI has offered both opportunities and challenges to regulatory agencies. Therefore, the presentation will also touch on the perspectives of using AI in regulatory science regarding (1) how to assess and evaluate AI-based products and (2) how to develop and implement AI-based application to improve the agencies' functions.

**Presentation:** Oral

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## Inter-laboratory variability in behavior-based severity assessment

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Evidence-based severity assessment is essential as a basis for ethical evaluation in animal experimentation to ensure animal welfare, legal compliance and scientific quality. To fulfil these tasks scientists, animal care and veterinary personnel need assessment tools that provide species-relevant measurements of the animals' physical and affective state.

In a three-center study inter-laboratory robustness of body weight monitoring, mouse grimace scale (MGS) and burrowing test were evaluated. The parameters were assessed in naive and tramadol treated female C57BL/6J mice.

During tramadol treatment a body weight loss followed by an increase, when treatment was terminated, was observed in all laboratories. Tramadol treatment did not affect the MGS or burrowing performance. Results were qualitatively comparable between the laboratories, but quantitatively significantly different (inter-laboratory analysis). Burrowing behavior seems to be highly sensitive to inter-laboratory differences in testing protocol. All locations obtained comparable information regarding the qualitative effect of tramadol treatment in C57BL/6J mice, however, datasets differed as a result of differences in test and housing conditions.

In conclusion, our study confirms that results of behavioral testing can be affected by many factors and may differ between laboratories. Nevertheless, the evaluated parameters appeared relatively robust even when conditions were not harmonized extensively and present useful tools for severity assessment. However, analgesia-related side effects on parameters have to be considered carefully.

**Presentation:** Oral

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## Monitoring of severity and implementation of refinement measures in DSS induced colitis in mice

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Animal models of colitis are used to explore the underlying pathogenesis of inflammatory bowel diseases (IBD) and to develop therapies for the treatment of IBD in humans. Human IBD patients report reduced well-being and pain, it therefore can be assumed that this condition is also burdening and painful in laboratory animals. When performing animal experiments for IBD research, particular emphasis should therefore be given to the monitoring of pain and distress as well as to the implementation of adequate refinement measures.

For a systematic analysis of the implementation and reporting of these refinement measures we performed a systematic literature review on the reporting of details of the DSS induced colitis model, measures to reduce bias and information on clinical monitoring, refinements and humane endpoints.

In general, reporting of many of these important aspects was poor, indicating that this research field has yet to adopt reporting guidelines such as ARRIVE, the Gold Standard Publication Checklist or the colitis methods checklist (Bramhall et al., 2015). While many studies used the common disease activity index (DAI) for monitoring of colitis progression, other available severity assessment tools were rarely used and refinement measures like analgesia were omitted in virtually all studies due to concerns of pharmacological side effects.

We conclude that there is a need for evidence based severity assessment and refinement of DSS induced colitis mouse models.

### Reference

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**Presentation:** Oral

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## Systemic toxicity predictions using in vivo and in silico approaches

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New approach methodologies to traditional animal testing are currently being developed and evaluated for use in chemical safety risk assessment. One important goal is to predict systemic



points of departure (PODs), such as BMDs or LOAELs without using animals. This talk will summarize methods being developed at the EPA using *in vitro* to *in vivo* extrapolation (IVIVE) and *in silico* QSAR methods. In the IVIVE approach, one measures the lowest *in vitro* concentrations at which bioactivity is seen using high-throughput screening assays or various high content approaches such as transcriptomics or cell imaging assays. These are converted to *in vivo* equivalent values using high throughput toxicokinetics methods. A comparison between the IVIVE and animal-based PODs shows that this IVIVE method is health conservative in most cases (90-95% of chemicals). New QSAR methods will also be presented both for directly predicting PODs and for predicting the IVIVE toxicokinetics parameters, which are typically the most difficult to measure *in vitro*. Because of the health protective and high-throughput properties of these methods, they are ideally suited for initial screening and prioritization applications in chemical risk assessment. This work does not reflect the official policy of the US EPA.

**Presentation:** Oral

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## Towards virtual control groups for animal toxicity studies – An eTRANSAFE initiative

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Sharing legacy data from *in vivo* toxicity studies offers the opportunity to analyze the variability of control groups stratified for strain, age, duration of study, vehicle and other experimental conditions.

Historical animal control group data may lead to a repository, which could be used to construct virtual control groups (VCGs) for toxicity studies. VCGs are an established concept in clinical trials (Eichler and Sweeney, 2018), but the idea of replacing living beings with virtual data sets has so far not been introduced into the design of animal studies.

Given the fact that toxicity studies usually consist of three dose groups plus one control group, the use of VCGs has the potential for a 25% reduction in animal use. Provided regulatory acceptance can be achieved, this would represent the biggest reduction initiative in pharmaceutical toxicity testing.

Prerequisites for such an approach are the availability of large and well-structured control data sets as well as thorough statistical analyses. The IMI projects eTOX and eTRANSAFE have laid the foundation for data sharing among the pharmaceutical industry. Efforts are now being undertaken to share control animal data also from confidential data sets. Since control animal

data are not related to the drug candidate and thus pose no IP issues, control group data can be shared without restrictions.

Participating companies have started to collect control group data for subacute (4-week) GLP studies with Wistar rats (the strain preferentially used in Europe) and are analyzing these data for its variability. In a second step, the control group data will be shared among the companies and cross-company variability will be investigated. In a third step a set of studies will be analyzed to assess whether the use of VCG data would have influenced the outcome of the study compared to the real control group.

### Reference

Eichler, H.-G. and Sweeney, F. (2018). *Clin Trials* 15, Suppl 1, 27-32. doi:10.1177/1740774518755058

**Presentation:** Oral

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## Establishment of a developmental neurotoxicant screening using SOX1-GFP mouse embryonic stem cells

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Developmental toxicity tests have been made by embryonic stem cell tests at the European Centre for the Validation of Alternative Methods or by embryonic body test in our laboratory (Hong et al., 2015). However, no neuronal-specific developmental toxicity test has been made yet. Therefore, this study was carried out using a Sox1-GFP mouse embryonic stem cells to exploit the developmental neurotoxicity test. The expression of Sox1, a marker for neural progenitor, can be detected by green fluorescence and the fluorescence density is a critical factor to achieve neuronal differentiation (Li et al., 2009). Sox1-GFP mouse embryonic stem cells were treated for 24 hours with 5-fluorouracil, bisphenol A, chlorpyrifos, clioquinol, diazinon, hydroxyurea, lead acetate and nicotine as developmental neurotoxicants, or saccharin, sodium bicarbonate, sodium gluconate, and penicillin G as non-neurotoxicants. CCK-8 assays were performed to determine IC50 values after 48 hours of chemical treatment. The fluorescence intensity of GFP was measured after 4 days of treatment with cells using an automated digital microscope. Through CCK-8 assay, IC50 values of developmental neurotoxicant chemicals were obtained, whereas non-neurotoxicant chemicals showed low effects (Liang et al., 2019). In addition, the fluorescence intensity of GFP was not decreased with non-neurotoxicants. However, neurotoxicants decreased the fluorescence intensity of GFP at higher concentrations. This decrease of fluorescence intensity indicates that the neuronal differentiation of Sox1-GFP mouse embryonic stem cells is inhibited by the chemicals. Taken together, this study produced a mod-



el of the developmental neurotoxicity tests used embryonic stem cells that may use to evaluate the toxicity of new chemicals or new candidate drugs.

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**Presentation:** Oral

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## Phenotype-based mechanistic studies for the assessment of drug safety and drug-drug interactions in zebrafish: Efficacy of dietary supplements

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Zebrafish embryos are routinely used in chemical toxicity assessments and are considered excellent pre-clinical models. For mechanistic studies, zebrafish embryos are better suited than higher order vertebrates since the embryos can be treated with a number of drugs at once and the phenotypic changes in response are quick and visible. In our studies, in addition to organ-specific toxicities, manifestations of drug-drug interactions arising from the use of combination drugs can be monitored *in vivo* in zebrafish. Live monitoring of multiple organ/tissue toxicities, such as cardiotoxicity and neurotoxicity, is an advantage since the embryos/larvae are transparent. Analyses of key enzymes involved in drug metabolism, such as the cytochrome P450 family (CYP) members, help further the understanding of the role of drug metabolism in the expression of various toxicities. Several drugs, such as ketamine, verapamil, and cyclosporine A show effects (alone or in combination with other drugs) in these embryos that are similar to those in humans. Importantly, the modes of action using phenotypic, biochemical and genomic approaches can be elucidated using these embryos. Subsequently, such findings have enabled the prevention of the adverse effects by co-treatment with dietary supplements (e.g., acetyl L-carnitine and N-acetylcysteine). Our studies provide clues as to how the adverse outcomes of these drugs might occur in humans, thus leading to better risk

characterization and assessment. Furthermore, potential therapeutic intervention points are also revealed as the effector molecules of their actions are precisely identified.

**Presentation:** Oral

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## E-learning resources to support training for project evaluation, project and procedure design, and severity assessment framework

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Effective and consistent implementation of EU Directive 2010/63 requires appropriate training of all those with responsibilities for the use of animals in research. Although training of those conducting procedures has been implemented with moderate success, other areas of training have shown less consistency. In particular, training of those with responsibilities for the Project Evaluation process has yet to be established in some member states, and the availability of training courses in study design varies considerably.

A key element of the Directive is the assessment of severity, however no specific training module and learning outcomes have been developed for this critical area.

Developing such training is challenging and resource intensive. To provide resources to support all those involved in the process, and to encourage a consistent approach amongst member states, the Commission is supporting the development of a series of E-learning programs. These will support training in Project Design (EU10 and 11), Project evaluation (EU 25) and in Severity Assessment (EU 12).

Learning outcomes for the new module in Severity Assessment have been developed. Three linked modules to support training in this area, together with modules for EU10, 11 and 25 have undergone expert external review and user-testing. Final versions of the modules (9 in total) were launched in early 2021 and are freely available via the ETPLAS web platform.

We consider that the European Commission's provision of these modules will encourage a more consistent approach to the development and evaluation of Project applications and promote harmonization of the harm:benefit analysis. One of the key goals in our development of these resources is to make them accessible to all those with responsibilities in these areas and to encourage incorporation of all three "Rs" into the design and evaluation of studies that involve the use of animals.

**Presentation:** Oral



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## Interactive discussion – How are we doing?

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Directive 2010/63/EU requires that every establishment using, breeding or supplying animals for research and testing has an Animal Welfare Body (AWB). The AWB has a number of tasks relating to setting up and reviewing operational processes that have a bearing on animal welfare, following the development and outcome of projects, and rehoming animals. Two especially important AWB roles are to advise on animal welfare and the Three Rs.

This interactive discussion session will allow participants to reflect on how effectively different countries and institutions have implemented the AWB, including what is working well; whether there are outstanding challenges and how these could be addressed; and what kind of networks have been established in different countries to help communicate and share good practice between AWBs.

**Presentation:** Oral

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## Avoiding mortality during procedures

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This presentation will provide an overview of approaches to avoiding unexpected mortality in animals undergoing regulated procedures, as set out by a multidisciplinary working group convened by the RSPCA, UK Laboratory Animal Science Association (LASA) Laboratory Animals Veterinary Association (LAVA) and Institute of Animal Technology (IAT) (Hawkins et al., 2019).

Avoiding mortality was addressed as part of the RSPCA-led “Focus on Severe Suffering” initiative. This is because actual severity is assumed in EU law to be “severe” if an animal is found dead, and the death is likely to be due to the procedure (unless an informed decision can be made that the animal did not suffer severely before death). The full resource addresses three main categories of mortality:

- unpredicted mortality in stock animals held for future breeding or experimental use;
- predictable mortality of animals, e.g., in some regulatory toxicology studies; and
- unexpected mortality in animals undergoing procedures.

The talk will focus on avoiding mortality during procedures, providing examples of approaches including reviewing welfare assessment, undertaking pilot studies, improving staff training, in-

vesting in animal monitoring and structured review of records. This can create some issues that need further consideration, e.g., increased monitoring of animals can cause harms; additional resources may be required; and indicators may be more difficult to interpret in aged animals. Local committees, such as Animal Welfare Bodies, Animal Care and Use Committees and ethics committees, can help to implement initiatives to avoid mortality and address any wider ethical implications that may arise.

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**Presentation:** Oral

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## The Animal Welfare Body – How are we doing

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In the presentation “The Netherlands AWB network” a reflection on the activities of the AWBs in the Netherlands, the national AWB platform, and its role in discussions with the National Center of advice on animal experiments (NCAD), the Dutch Authorities for Scientific Procedures on Animals and the Transition of Animal Free Innovation will be presented. This presentation is part of the AWB workshop and will inform about the initiatives of the AWB platform to promote the 3Rs and specifically the efforts on alternative methods.

**Presentation:** Oral

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## Exploiting safety data shared by pharmaceutical industry: The eTRANSAFE project

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The pharmaceutical industry is one of the sectors generating comprehensive high-quality safety assessment data. Recently, the value of these data has been recognized and different ini-



tiatives have started to mine this valuable resource (Sanz et al., 2017). The process for exploiting legacy safety assessment data starts with their collection from multiple sources but requires the application of knowledge management and integration tools before it can be used for practical purposes. Here we will introduce eTRANSafe, an ongoing IMI2/JU funded project bringing together 12 pharmaceutical industries, 6 SMEs and 8 academic institution for collecting and exploiting this kind of data.

eTRANSafe is developing an integrative data infrastructure supporting the application of computational methods and tools. The data collected can be retrieved, visualized, and analyzed in multiple ways to answer multiple relevant questions. One of the most ambitious goals is to use this information to improve the reliability of translational drug safety assessment. With this aim, the project is collecting both preclinical and clinical data and mapping the information collected in either domains. The knowledge platform being developed by the eTRANSafe project contains all the data in an integrated form as well as advanced tools for data extraction, visualization and analysis. Beyond the simple collection of findings, the project is incorporating mechanistic information as a key strategy to improve the translatability between species and better understand the ability of preclinical studies to predict clinical outcomes. Eventually, such a systematic approach might contribute to the avoidance of animal studies lacking predictivity. In addition, the data will be further exploited with the help of machine learning and deep learning methods for translating knowledge into mathematical models and produce reliable predictions for novel compounds.

*Funding: This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (IMI2/JU) under grant agreement No 777365 (eTRANSafe).*

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**Presentation:** Oral

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## ReCAAP: Carcinogenicity waivers for food-use pesticide registration

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Reviews of the rodent cancer bioassay over the past 40 years have raised questions about its relevance and regulatory need to assess risk to human health. As a result, a working group comprised of experts from government, industry, and non-government organizations have formed the Rethinking Carcinogenicity

Assessment for Agrochemicals Project (ReCAAP) to evaluate the appropriateness of waiving rodent bioassays for the registration of food-use pesticides. This presentation will provide case study examples of carcinogenicity waivers formulated through a weight of evidence-based (WoE) approach. The WoE includes information on pesticide exposure, mode-of-action, physicochemical properties, and sub-chronic toxicological data from defined endpoints. This effort has established criteria for when the mouse and/or rat cancer bioassay can be waived while ensuring that pesticide human health risk assessments are protective.

**Presentation:** Oral

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## Enabling chemical substance data integrative analysis and applications

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The publicly accessible chemical databases generally associate chemical substances with single structures and do not provide easily accessible data on substance identity and composition. Cefic's Long-Range Research Initiative (LRI) AMBIT software enable the representation of chemical substances in real industry conditions. The AMBIT tool is available online and is loaded with non-confidential REACH data supplied by the European Chemicals Agency (ECHA). The AMBIT data model also supports nanomaterials, and enabled integration of large data sets of nanosafety data through eNanoMapper database. Experiences and approaches towards data harmonization and data interpretation in the context of diverse data sources are presented. AMBIT user friendly web interface and REST API enable web browser and programmatic access to the data, laying the foundation towards multifaceted analysis through modern machine learning methods and building specific toxicology application as read-across.

**Presentation:** Oral

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## IATA as an opportunity for next generation risk assessment

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Safety assessment of cosmetics can no longer be performed by generating new animal studies in Europe. To address this, initiatives based on the use of new approach methodologies (NAMs) in expo-



sure-centric and hypothesis driven approaches are led by the cosmetic sector. The purpose of the assessment is to be protective of human health as stated in the principles developed by the International Cooperation on Cosmetics Regulation (ICCR, Dent et al., 2018). In line with these principles, an Integrated Approach to Testing and Assessment (IATA) from the European framework project – SEURAT1 – (Berggren et al., 2017; OECD Series on Testing & Assessment No. 275, 2017) was utilized in a next generation risk assessment case study. The case study focused on the use of propylparaben as a preservative in cosmetics. The approach in this case study entailed a “learning by doing” exercise which was designed to assess the value added by NAMs in safety assessments based on read-across. The objectives were to test the methodology and evaluate how data can be applied in decision making. The problem formulation in this assessment was an assumed data gap for reproductive toxicity for propylparaben (available experimental animal reproductive toxicity data were purposefully not taken into consideration). A tiered approach was employed to assess the reproductive toxicity hazard associated with dermal exposure to propylparaben in cosmetics. NAMs were utilized to support the read-across hypothesis and to inform human-relevant risk assessment and support decision making. This NAM-based IATA approach facilitated testing or modification of read across hypotheses and supported the assessment of analogue chemical(s). Efforts are currently underway to continue to evolve read across in this direction. The case study was led in collaboration by a working group of members of the Cosmetics’ Europe Long Range Science Strategy – LRSS – and the Horizon 2020 EU-ToxRisk consortium.

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- OECD IATA Case Studies Project, Series on Testing & Assessment No. 275. ENV/JM/MONO(2017)27 Chemical safety assessment workflow based on exposure considerations and non-animal methods.

**Presentation:** Oral

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## Building an AOP-driven defined approach guideline

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Skin sensitization provides a rare example where 1) the full AOP has been described, and 2) multiple validated *in vitro* methods ex-

ist to measure all key events in the AOP, though no single *in vitro* method has been deemed adequate for replacing the animal data requirement to date. Driven by regulatory restrictions on animal testing for cosmetics ingredients, multiple in-house integrated approaches to testing and assessment (IATAs) emerged. As the information sources and data interpretation procedures become fixed, the IATA can evolve into a Defined Approach (DA) and may be harmonized and covered by the OECD agreement on the Mutual Acceptance of Data. Based on an evaluation of the performance of DAs against a large set of animal and human reference data, the DAs may be more predictive of the human response than the “gold standard” animal tests. This would be the first internationally harmonized guideline that uses alternative approaches in combination to replace an *in vivo* data requirement for regulatory purposes. This is also a model of how future guidelines for more complex endpoints may be developed.

**Presentation:** Oral

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## A North American regulatory perspective on skin sensitization risk assessment

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Numerous non-animal alternatives for skin sensitization assessment have been developed and are at various stages of evaluation (Ezendam et al., 2016). Because skin sensitization is a complex process, it is unlikely that any individual alternative method will completely replace current animal tests, and even the *in vitro* and *in chemico* methods that have been adopted as international test guidelines are not yet recommended as stand-alone replacements for animal test methods. Thus, a number of approaches to integrate the information from multiple non-animal methods as a way to overcome the limitations of individual tests and more accurately assess the potential for skin sensitization have been evaluated and compared to one another (Kleinstreuer et al., 2018). These approaches, which preclude the use of expert judgement by applying fixed data interpretation procedures to specific data streams, are referred to as “defined approaches” or “DAs.” These DAs use combinations of non-animal tests that align with key events in the adverse outcome pathway for skin sensitization. Certain DAs go beyond the binary and categorical hazard/potency predictions and provide quantitative information that can be used to derive points of departure for risk assessment applications. Their application is being investigated by regulatory authorities and will be discussed here.

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**Presentation:** Oral

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## Applying *in vitro* to *in vivo* extrapolation to NAM-derived PODs

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*In vitro* to *in vivo* extrapolation (IVIVE) enables estimation of an equivalent administered *in vivo* dose based on an *in vitro* activity concentration. IVIVE facilitates risk assessment applications for new approach methodologies (NAMs) by relating *in vitro* assay activity concentrations, such as points of departures (PODs), to potential *in vivo* exposures and observed effects. Many tools for IVIVE analysis are neither freely available nor easy to use by individuals with little computational training. To address this challenge, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) has developed an online tool for IVIVE analysis that is publicly available through the Integrated Chemical Environment (ICE). Leveraging the US EPA's high-throughput toxicokinetics (httk) models, the ICE IVIVE tool brings together curated and annotated *in vitro* data, quantitative structure activity relationship (QSAR) predictions for key parameters, and pharmacokinetic (PK) models of varying complexity to predict corresponding *in vivo* exposures. Users can select *in vitro* data housed in ICE or input their own data for IVIVE analyses. Available PK models for various exposure routes include a one-compartment PK model and multi-compartment physiologically based PK models, parameterized with experimental or QSAR-predicted values for plasma protein binding and intrinsic clearance. In summary, the ICE IVIVE tool is accessible to a diverse stakeholder community and provides an optimized approach for using *in vitro* data to quantitatively predict *in vivo* effects.

**Presentation:** Oral

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## The effect of mechanical loading in a bone-on-a-chip

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Conventional 2D cell culture, although of immense value in research, is strongly limited in recreating tissue-specific parameters. One possible way to overcome these shortcomings without relying on animal testing is the use of microfluidic organ-on-chip systems to recreate physiology of the tissue of interest and to co-culture different cell types in 2D or 3D (Kodzius et al., 2017). Bone is characterized by a constant resorption and formation of new tissue in response to external cues such as mechanical load, a process termed remodeling (Scheinpflug et al., 2018). Therefore, a bone-on-a-chip system that enables mechanical loading of a 3D organoid was established in this project. In a initial step, it was assessed whether application of a cyclic mechanical loading protocol (1Hz, 10% strain) is osteoinductive in our bone-on-a-chip system. For this, a collagen scaffold loaded with osteoblasts (OBs) is cultured for seven days under mechanical load and perfusion. To investigate energy metabolism and osteogenic activity, daily sampling of culture media and endpoint analysis of the cell-laden scaffold were performed. Mechanical loading in the bone-on-a-chip indeed induces osteogenesis in OBs when compared to unloaded cells. This is demonstrated by an elevated activity of alkaline phosphatase (ALP) and an increase in extracellular inorganic phosphate. Furthermore, the transcription of the osteogenic marker RUNX2 is elevated when mechanical loading is applied. OBs that undergo osteogenesis, perform additional aerobic glycolysis to match their high energy demand and also provide pyruvate as a precursor for collagen synthesis. Elevated levels of lactate and an increase in glucose consumption was detected, while the actual metabolic activity in the mitochondria was not affected upon mechanical loading, indicating additional aerobic glycolysis. It can therefore be concluded that the implementation of defined and dynamic mechanical loading in the bone-on-the-chip was successful.

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**Presentation:** Oral



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## Animal-free strategies in food safety & nutrition

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Substantial advances are being made in the development of non-animal based methods to assess safety (specifically toxicity) and beneficial effects of foods and food ingredients, including QSARs, omics and organs on chips. In the European Union, providing evidence on safety or health effects is key before new products or claims are allowed on the market. The scientific evidence, generated by the food business operator, is reviewed by the European risk assessor EFSA, the European Food Safety Authority. EFSA's scientific opinion is one of the most important factors for the European Commission to allow or deny the product or claim on the market. Whereas the submitted scientific evidence often relies on animal tests, these alternative methods to assess safety or health benefits have great potential for being used in these regulatory scientific assessments. This study shows to what extent the current legal requirements in Europe for scientific assessments related to food production give room for implementing these novel methods. Secondly, promising alternatives to animal testing for safety and health benefit assessments in foods are reviewed, focusing on both relatively straightforward approaches that can be used in risk assessment already now but also identifying new alternative strategies and approaches. This study highlights that various alternatives have future potential for being incorporated in scientific assessments for foods, but also identifies methods which can be employed in these safety and health benefit evaluations already.

**Presentation:** Oral

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## Cosmetic Europe case studies exploring alternatives to systemic toxicity testing

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Cosmetics Europe case studies have used new approach methods (NAMs) in various ways to explore the risk assessment of cosmetic relevant substances, where animal testing is no longer permissible nor desirable. Case studies play a key role in exploring the value of NAMs in different types of safety assessment and the repeated dose systemic toxicity case studies are examples of where NAM has been used in different ways, depending on the question that needs to be answered.

In Read Across, untargeted and targeted NAMs have helped to strengthen the identification of suitable substances for read across, increasing confidence in the NOEL used as a POD for the risk assessment. NAMS have also been used to inform on relative potency of analogue mode of action and to predict internal exposure in both human and animal studies allowing for a risk assessment approach based on internal exposures of the human versus the animal study.

In a laudable effort to move away from the use of animal data to assess safety of a new cosmetic ingredient a broad screening battery of NAM are being explored to identify a POD for the ab initio risk assessment as well to characterize the internal dose metrics associated with a given exposure scenario. This approach offers promise for well-defined exposure scenarios but in absence of a deeper understanding of modes of toxicity and how interactions at the molecular level can lead to adverse outcomes it still remains a grand challenge to move away from reliance on animal data in any shape or form in the field of repeated dose systemic toxicity.

**Presentation:** Oral

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## Hinduism and cultural differences and similarities in thinking about animal welfare and the use of animals in science

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Animal welfare often means different things to different people, and opinions are varied and debates often heated and emotional and sometimes have political innuendoes. These Cultural difference plays a major role in animal welfare in Hinduism as we are



grown up considering animal as a form of God and the principle of AHIMSA (Non-Violence) The concept of AHIMSA first appeared in the Rig Veda (ancient Indian collection of Vedic Sanskrit hymns) 3000 BCE – RIG Veda (X.87.16). No person should kill animals who are helpful to all. By serving them, one should obtain heaven 2500 BCE –Yajur Veda (XII.47). Born of thee, on thee move mortal creatures. Thou bearest them –The biped and quadruped thine, O earth, are the five races for whom the sun, as he rises, as he rises, spreads his rays. (2000 BCE – Atharva Veda (XII.1.15).

As late as in 1600 there was a Dabra Pinjarapole (Cow shelter) which was an inspiration for RSPCA in 1824 followed by Calcutta Society for the Prevention of Cruelty to Animals. Thereafter passing of the Prevention of cruelty to an animal in 1960 and wildlife protection act 1972. So also in 1976 article 51a(g) “It shall be the fundamental duty of every citizen of India to protect and improve the natural environment including forests, lakes, rivers, and wildlife, and to have compassion for all living creatures”. 1986 computer-aided dissection introduced in India. A series of steps taken from 1996 to 2002 for animal welfare including the formation of the Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA) implements guidelines governing the use of animals in research.

So cultural difference plays a vital role in animal usage which is evident from the issue raised until the Supreme Court of India.

**Presentation:** Oral

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### 3R initiatives of the pharmaceutical industry

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The European Federation of Pharmaceutical Industries and Associations (EFPIA) represent the pharmaceutical industry operating in Europe. Through its direct membership of 36 national associations and 39 leading pharmaceutical companies and Partners in Research including CROs, we are involved in a number of initiatives, which affirm the key principles of the 3Rs and support animal welfare. As part of this commitment, the European pharmaceutical industry promotes these initiatives internally and externally.

The presentation will give some insight into how the pharmaceutical industry collaborates within the research field recognizing how the sector operates to meet the requirements to effectively implement the requirements of Directive 2010/63/EU and also where researchers and technicians go beyond regulatory requirements to develop practices leading to improved animal welfare and focused 3Rs efforts in everyday practice. The presentation will include information on how we continue to work with regulators to ensure the fastest possible uptake of new approaches that balance increased effectiveness in safety and efficacy assessment and impact on 3Rs and how we engage all stakeholders and

disciplines in dialogue and collaborations and facilitate exchange of good practice between life science community stakeholders to improve animal welfare and scientific outcome by addressing emerging animal welfare issues and mechanisms that share practices that promote good science, animal welfare and the 3Rs.

**Presentation:** Oral

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### 3D scaffold-based neuroblastoma model for evaluating cytotoxic and miRNA-targeted therapeutics

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Neuroblastoma is an aggressive paediatric cancer of the sympathetic nervous system. Current therapies are not effective in the long term for almost 80% of patients with clinically aggressive high-risk disease.

The accurate representation of the tumour architecture and patient diversity are two primary challenges for paediatric drug development, particularly with the limited number of patients eligible for clinical trials (Nolan et al., 2020).

We aimed to bridge the gap between conventional 2D culture and *in vivo* tumours for neuroblastoma by developing a 3D tissue-engineered platform and exploring its therapeutic relevance to genotoxic and targeted drugs.

Chemotherapeutic sensitive Kelly and resistant KellyCis83 neuroblastoma cell lines were cultured in a 3D *in vitro* on collagen-based scaffolds containing either glycosaminoglycan or nanohydroxyapatite and compared to 2D cell culture and an orthotopic murine model (Piskareva et al., 2015). Cells actively infiltrated and proliferated over the 21-day timeframe and exhibited physiological activity by secreting CgA demonstrating the correlation between cell numbers and concentration of CgA. Both cell lines responded to cisplatin, a cytotoxic drug commonly used in neuroblastoma treatment, displaying > 100-fold increased resistance to cisplatin treatment when compared to 2D cultures, exhibiting chemosensitivity similar to orthotopic xenograft *in vivo* models (Curtin et al., 2018). The data is in agreement with previous studies reporting a significant increase in cancer cell resistance to chemotherapy when grown in 3D when compared to their 2D monolayer counterparts in various cancer types. The efficacy of cellular uptake and gene knockdown by liposomes bearing miR-324-5p was similar in both 2D and 3D *in vitro* culturing models highlighting the proof-of-principle for the applicability of this model for validation of miRNA function.

We successfully established and characterised a physiologically relevant, scaffold-based 3D neuroblastoma model, strongly sup-



porting its potential value in the evaluation of chemotherapeutic and miRNA-based drugs and investigation of neuroblastoma biology.

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**Presentation:** Oral

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## Cell sheet-based myocardial tissue engineering for animal alternative testing

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Recent advances in stem cell biology and tissue engineering are realizing human tissue model fabrication. Tissue-engineered functional models using human iPS-derived cells have the potential as animal alternatives. Now various types of tissue engineering technologies have been developed. They include scaffold-based tissue engineering, bioprinting and spheroid usage. On the other hand, as one of the tissue engineering technologies, we have developed "cell sheet-based tissue engineering" which utilize cell sheets harvested from temperature-responsive culture dishes. We have already succeeded in fabricating 3-D beating human cardiac tissues by using iPS cell-derived cardiomyocyte sheets. Macroscopically beating cardiac tissues has enabled direct measurement of contraction force. When the constructs are stretched, contraction force increased, which was consistent with Frank-Starling Law. both the contractile force and beating rate were significantly increased by the administration of adrenaline, which are the physiologically relevant responses for cardiac tissues. Several anti-cancer drugs decreased the contraction force as seen in clinical practice. We have also developed tubular and dome-like myocardial tissues. The beating of the tissues has evoked inner pressure which are successfully measured in real-time. These human myocardial tissues should be useful as a new evaluation system for cardiotoxicity and cardio pharmacology. Thus, cell sheet-based myocardial tissue engineering has great potential to engineer functional 3D cardiac tissues which might contribute to animal alternative testing.

#### Reference

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**Presentation:** Oral

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## A machine-learning and systems biology strategy requires systematic review to develop animal replacement alternatives

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As an alternative to animal models of human disease, a systems biology format has been proposed with machine-learning algorithms central to identifying and predicting human data patterns associated with disease outcome (1). While primarily aimed at biomedical research, a system has been also proposed for "cell-free" *in vitro* toxicology (2). While validated predictions and associations in a range of human data can be identified, systematic reviews are required to support the ultimate development of this system's framework into a validated animal replacement alternative.

Based on predictive data patterns, systematic reviews (SR) will be designed to investigate the identified compounds and processes identified by machine-learning (M-L) at a deeper level, with meta-analyses further augmenting these data pipelines. Results from SR can then be used to design further M-L experiments, potentially leading to -OMICS investigations on human volunteers, to augment the breadth of data available for modelling.

Since M-L generates predictions and associations, SR will be crucial to determine mechanisms that underpin the disease/condition of interest, which are required for eventual therapeutic translation.

In addition to laboratory and clinical data for M-L, this presentation and session will consider the benefits of text-mining as a tool to enhance the SR process – For example, by identifying concepts in the literature that guides the translation of research results generated from animal-free models of human disease.

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**Presentation:** Oral



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## Integrated research and testing strategy to go beyond the 3Rs

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As a diversified global healthcare leader focused on patients' needs, Sanofi is morally and legally obligated to ensure the quality, safety and efficacy of its medicines, vaccines, medical devices, and consumer healthcare products. Besides the regulatory requirements, the responsible use of animals is essential in the research and production process. Animals remain a small but an integral part of a comprehensive research and testing strategy that includes non-animal methods (such as computerized models and *in vitro* testing) and clinical research. The standard approach is designed to use animals only when a non-animal method is not suitable for the required use or not accepted by the authorities (replacement), with the smallest number necessary for quality science (reduction) while implementing state-of-the-art practices to promote animal welfare and prevent pain and distress in housing, procedures and treatment (refinement). To go further, a strategy, relying on regulatory sciences, translational medicine and breakthrough innovation, has been developed to increase the proportion of non-animal methods, including clinical research, to reduce significantly the necessity to use animals in research and production. Several decades ago, the development on new drugs and vaccines mainly relied on animal studies. Nowadays, all the projects require and use non-animal data, *in vivo* studies, patient data and clinical research to assess the safety and the efficacy of new drugs. We strongly believe that, based upon the development of regulatory sciences, translational medicine and innovation, reduction of the ratio animal studies versus non-animal methods is effective and it will accelerate.

**Presentation:** Oral

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## Advancing carcinogenicity assessment: A novel methodological approach to integrate information and further the impact on the 3Rs

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Cancer is a key public health concern, being the second leading cause of worldwide morbidity and mortality after cardiovascular diseases. While it is difficult to establish the contribution of

chemical exposure to the societal burden of cancer, a number of measures can be taken to better assess the carcinogenic properties of chemicals and manage their risks.

The presentation aims to discuss how these measures can be informed, not only by the traditional data streams of regulatory toxicology, but also by using new toxicological assessment methods (Madia et al., 2019). In this context, the JRC started investigating ways to exploit existing data to further the impact on the 3Rs for carcinogenicity assessment. In a newly developed methodology, toxicity test methods are dissected in a reverse-engineered manner to allow the systematic organization of the overall information across endpoints to the key characteristics of carcinogens. The expected outcome is a set of options that are motivated by a mechanistic understanding of the toxicological effects and their inter-relationships for waiving redundant testing and ultimately minimize reliance on apical endpoint tests. It will also allow building a toxicological narrative chemical- and/or disease-specific whilst providing a flexible and resource-efficient tool for carcinogenicity assessment.

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**Presentation:** Oral

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## Development of metabolically competent human and rat spheroid models and application of high-throughput transcriptomics towards 3Rs strategy

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Cells cultured as three-dimensional spheroids exhibit physiologically-relevant xenobiotic metabolism competence and tissue-like functionality. Integrating conventional *in vitro* measurements with high-content screening assays such as high-throughput transcriptomics (HTT) would enable systematic characterization of chemical induced effects on biological systems. We have developed spheroid liver models of a human progenitor cell line, HepaRG, and primary rat hepatocytes. Activities of xenobiotic metabolism enzymes, CYP1A2, CYP2B6 and CYP3A4 were 2 to 20-fold higher in HepaRG spheroids compared to the sandwich-cultured primary human hepatocytes. Similarly, specific activities of CYP1A2 and CYP3A enzymes in rat spheroids re-



mained at the levels as freshly isolated hepatocytes even after 14-days in culture. High-throughput gene expression analysis using the S1500+ gene set was performed on HepaRG spheroids treated with ten concentrations of a reference set of chemicals in repeated exposure regimens. Baseline gene expression and biological pathway responses showed significantly higher enrichment scores in spheroids compared to monolayer cultures. Exposure to aflatoxin B1 and benzo(a)pyrene showed activation of genes and pathways related to their metabolism and anticipated downstream signaling events such as cell cycle, p53 signaling, DNA damage and cancer. BMDEExpress was used to calculate point of departure for individual genes and pathways associated with concentration-related molecular perturbations. Biologically relevant pathways associated with exposure of chlorpromazine and valproic acid were activated at sub-lethal concentrations. Differences in gene and pathway-level expression between liver injury chemicals (trovafloxacin, troglitazone and tolcapone) and their structurally similar analogs was observed. The initiation of molecular events associated with chemical exposure and progression of events that lead to adverse outcome effects were clearly evident with high-throughput transcriptomics analysis. *In vitro* models utilizing spheroids and data-rich approaches may offer better resolution in identifying molecular level perturbations of biological systems and prediction of hazardous substances in support of replacement, reduction, and refinement of animal testing

**Presentation:** Oral

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## lncRNAs as novel source of diagnostic applications for early Alzheimer's disease and other dementia types – ADDIA Consortium and ADKIT Consortium

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There is an unmet need for an accurate, non-invasive biomarker test for the diagnosis of early Alzheimer's disease (AD). To identify new biomarkers, we focused on long non-coding RNAs (lncRNAs) as they are tissue-specific to identify lncRNA panel candidates for diagnostic of early Alzheimer's disease (AD) and other dementia types.

**Methods:** We performed a screening using NGS RNA-Sequencing to quantify over 127000 lncRNAs in human post-mortem brain tissue and blood samples including whole blood, PB-MCs and, plasma samples collected in prospective clinical stud-

ies that recruited patients with early AD, patients with late AD, patients with one of the other 5 dementia types and healthy controls.

**Results:** We identified for the first time several panels (i) a panel of brain-enriched lncRNAs never described before, (ii) panels of brain-enriched lncRNAs that are either expressed in whole blood or circulating in plasma. (iii) Interestingly, out of these, we also identified panels of lncRNAs that are differentially expressed in the blood of patients with AD or with other dementia types as compared to healthy control subjects.

The most accurate lncRNA panel to detect early AD is selected for use as Research-Use-Only test and is being further validated to compile the data dossier for submission to regulatory agencies for approval as an *in-vitro* diagnostic (IVD) tool diagnostic of AD. Additional clinical applications are for prognostic or therapeutic purposes.

**Conclusion:** Our results from studies combining the use of high-quality samples from well-designed prospective clinical studies, cutting edge technologies and scientific knowhow enabled to translate novel brain specific lncRNA panels measurable in blood as new non-invasive and accurate diagnostic approaches using highly specific and low represented sequences specific to neurodegenerative diseases.

**Presentation:** Oral

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## Air-liquid interface exposure for inhalation testing: Case studies

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There is a demand to implement *in vitro* alternatives for inhalation testing using human relevant lung cell models, realistic air-liquid interface (ALI) exposure systems, and proper dosimetry techniques to increase the predictability and accelerate the shift from *in vivo* towards *in vitro* testing. Presented here are case studies. In a first case study, BEAS-2B cells were exposed to various concentrations (0.72, 25, and 85 ppm) of triethoxysilane vapor using a capillary dosage unit coupled to a VITROCELL® 6/4 module. A significant concentration-dependent decrease in cell viability and increase in cytotoxicity was observed after ex-



posure to triethoxysilane and nitrogen dioxide (NO<sub>2</sub>, positive control) as compared to clean air (CA, negative control). A significant increase in expression of inflammatory markers was observed. Additional work is underway to test other silanes to determine if this *in vitro* system can detect the decrease in toxicity that correlates with increasing carbon-chain length and to determine the advantages of using a 2D cell line versus a 3D human reconstructed tissue model. Another case study is focused on ALI inhalation testing of petroleum-derived substances. A generation facility was successfully developed at VITO to volatilize ethylbenzene (EB) and VITROCELL<sup>®</sup> 24/48 exposure system was optimized and validated for CA, NO<sub>2</sub>, and EB exposure. A significantly decreased mean cell viability of 86%, 77%, and 47% was observed for exposure of A549 cells to EB of about 30,000, 40,000, and 50,000 mg/m<sup>3</sup>, respectively. Inflammatory and oxidative stress markers were evaluated as well. The difference between *in vivo* absorption and *in vitro* deposition (chemically determined) is a crucial element when setting an ALI dose-range. Quantitative *in vitro* to *in vivo* extrapolations from *in vitro* air concentrations applied for testing cell viability to *in vivo* air concentrations may be a promising method for screening acute adverse inhalation effects.

**Presentation:** Oral

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## Blood vessels in organs-on-chips

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Organs-on-chips are uniquely different from other cell culture models in that they are based on controlled microenvironment engineering. Therefore, they can capture progressively more complex physiological functions without relying on uncontrolled and unpredictable cellular self-organization (Van der Meer et al., 2012). The controlled nature of organs-on-chips also allows the systematic “personalization” of various aspects of the model, so that it truly becomes a reflection of ourselves and may be applied in targeted drug development and precision medicine (Van den Berg et al., 2019; Peck et al., 2020). In this talk, I will explain how we are applying this concept of systematic personalization as we engineer blood vessels for integration in organs-on-chips.

For the engineering of blood vessels in organs-on-chips, there are a number of key aspects that can be controlled and personalized: blood and vascular tissue, vessel geometry, and properties of the flow. For example, we have used blood from patients on anti-platelet medication and have detected differences in thrombosis at arterial flow rates in vessels-on-chips. Moreover, we have used CT angiography imaging data to engineer controlled 3D vessel-on-chip geometries, and have used these to study the local risk of thrombosis. Finally, we are moving away from using primary cell material and have been focusing on engineering

vessels-on-chips based on human pluripotent stem cell-derived tissues.

The power of personalizing organ-on-chip systems and their integrated blood vessels is only beginning to be demonstrated. By staying close to the design philosophy of controlled microenvironment engineering, it will be possible to further improve the personalized and functional complexity of these systems in the coming years.

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**Presentation:** Oral

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## Waiving repeat dose studies while confidently protecting human health from exposure to agricultural chemicals

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The Environmental Protection Agency’s (EPA) Office of Pesticide Programs (OPP) regulates the use of all pesticide chemicals. Numerous toxicity studies in laboratory animals are required for pesticide registration; however, regulatory statutes provide the EPA with the flexibility to modify the actual data and studies required on an individual basis. To better protect human health and the environment, OPP is developing and evaluating new technologies to supplement or replace more traditional methods of toxicity testing and risk assessment. OPP has been making significant progress towards the adoption of integrated approaches to testing and assessment (IATAs) and the reduction of animal use in acute toxicity testing. OPP has also developed processes for waiving repeat dosing toxicity tests, while also evaluating the use of new approach methodologies (NAMs) to support pesticide registrations.

**Presentation:** Oral



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## Religion-based cultural influences on ethics, animal welfare and use of animals in science

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Buddhism as a religion promotes love, compassion and kindness towards all living beings thus promoting the concept “do not harm/make them suffer (‘avihinsa’). Buddhism facilitates mental and physical wellbeing of animals. The first precept makes people aware of killing of animals is a sin and all five precepts are to promote ethical conduct of humans. The concept of “cause and its effect” is the fundamental principle in Buddhism. In this context, science is an accepted phenomenon in Buddhism; however, as scientists we must take steps not to harm animals for the benefit of humans.

According to Hinduism cultural differences play a major role in animal welfare as we are grown up considering animal as a form of God and the principle of AHIMSA (Non-Violence). Series of steps taken from 1996-2002 for animal welfare including formation of Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA) to implement guidelines governing use of animals in research and enforcing penalties for non-compliance.

Researchers in Malaysia prone to identify the usage and toxicity of various herbal material which are abundant in the country using laboratory animals such as rodents, rabbits and zebrafish. Since Islam is the main religion of Malaysia, the Islamic views has influenced in various aspects of daily activities including scientific research. Therefore Islamic point of view has become a basic principle for the establishment of the humane practice in using laboratory animals in science.

Many religions promote good and compassionate animal care, it be domestic or wild. The current world uses lab animals to discover new drugs and test toxicities of existing ones. A healthy research program exists if all these religions continue to promote such compassion towards animals. Culture of care is one such educated care that can promote the care of animals.

**Presentation:** Oral

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## Introduction – Focus on severe suffering

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All laboratory animal suffering is a concern, but the RSPCA believes that ending “severe” suffering should be a top priority. There are a number of reasons to do this: (i) the ethical obligation to avoid or reduce suffering, (ii) the requirement to adhere to the 3Rs principles and (iii) the scientific benefits. It is widely acknowledged that good quality science goes hand in hand with good welfare, and that unalleviated suffering can introduce avoidable variation and reduce the power of experiments.

As a scientific animal welfare organisation with a high level of national and international liaison with scientific and regulatory communities, we have been able to establish an integrated programme of work aimed at reducing and ultimately ending severe suffering. We promote constructive dialogue between those who are involved in the use, care and regulation of research and testing to identify practical strategies to avoid, or reduce the impact of, severe models and procedures. Our approach is well supported by the scientific community and the UK Government.

Our pioneering initiative has so far included the organisation of two major international conferences, the convening of several expert working groups, and the production, publication and dissemination of a range of resources, including a dedicated web resource (<https://focusonseveresuffering.co.uk/>) to help reduce and ultimately end severe suffering.

This talk will highlight how the RSPCA, as a scientific animal welfare organisation, working with, rather than against, the scientific community has managed to improve implementation of the 3Rs. Practical examples of approaches to reduce severe suffering and a range of resources that we have produced will be presented.

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**Presentation:** Oral



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## Towards replacing the two-year bioassay with short-term NAMs: Genomic and nongenomic activation levels can identify rat liver tumorigens

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Traditional data sources for cancer hazard assessment are resource-intensive, retrospective, and not feasible for the vast majority of environmental chemicals. Incorporation of quantitative (non)genomic data from short-term rodent studies may adequately define protective activation levels for potential tumorigens as a bridge to move from current testing to greater reliance on *in vitro* assays. We hypothesized that gene expression biomarkers that measure the activation of the major molecular initiating events (MIEs) in rodent liver cancer adverse outcome pathways as well as liver weight to body weight (LW/BW) and clinical chemistry (ClinChem) endpoints exhibit chemical-independent activation levels beyond which cancer occurs, and the activation levels could be used together as a NAM to predict liver cancer. The hypothesis was tested by defining activation levels of gene expression biomarkers of liver cancer MIEs using training sets from the 77 and 86 chemicals in the TG-GATES and DrugMatrix datasets, respectively and testing them in a number of contexts. The biomarkers tested consisting of 7-113 genes included those that predict genotoxicity, cytotoxicity, and activation of AhR, CAR, ER, or PPAR $\alpha$ . Activation levels were calculated as the maximum values derived from exposures that do not lead to liver cancer. In all cases, clear threshold values could be identified that were consistent across training and test sets. Activation levels derived from the TG-GATES or DrugMatrix studies had predictive accuracies of 77-100% and were most predictive when applied to test sets of 7 d and 14 d treatments (100% and 99%, respectively). In addition, activation levels derived from just 12 genes (2/biomarker) as well as LW/BW and ClinChem endpoints exhibited high predictive accuracy (up to 94%). These findings support the idea that genomic and nongenomic changes measured after short-term exposures can be used to establish activation levels that are predictive of later-life outcomes.

*Disclaimer: This abstract does not reflect EPA policy.*

**Presentation:** Oral

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## Virtual models for human developmental toxicology driven by new approach methodologies

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Toxicity is mediated by perturbation of a network of physiological processes at the molecular, cellular, tissue, organ, and organism level. Quantitative *in silico* modeling of network dynamics is an important goal toward predicting toxicological hazard from chemical-biological interaction. Systems toxicology mapping of network structure-function would reveal threshold events at the toxicological tipping point between adaptive and adverse biology. These events can be tested in dedicated new approach methodologies (NAMs) focusing on the molecular and/or cellular scale. Concentration-dependent compound-induced effects from targeted *in vitro* studies fed into the network model provide toxicity prediction at the level of the intact organism. These systems models utilize the extensive knowledge that exists on biological control of morphogenesis and the conservation of key biological pathways and processes enabling integration of data on among diverse species, from small model organisms to human development. Current efforts towards *in silico* modeling of specific morphogenetic processes include, for example, neural tube closure. In a CEFIC-LRI sponsored project we described the biological pathway network underlying vertebrate neural tube closure, using a text mining tool applied to the open scientific literature. This network is the input for developing a 3-dimensional computational model for *in silico* testing of compound effects on neural tube closure. The principal idea is that by adapting parameter settings in the *in silico* model based on effects on gene expression observed in relevant *in vitro* cell assays, their consequences at the higher integration level of neural tube closure can be predicted. Basic information fed into these dynamic systems models include: defined cell types, the molecular signals they provide, their target cell types, and the nature of their responses to signals. In due course these models are expected to revolutionize toxicity testing, enhancing human relevance based on enhanced insight into mechanisms of toxicity.

*Disclaimer: This abstract does not reflect EPA policy.*

**Presentation:** Oral



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## An integrated approach alternative for screening reproductive, developmental and endocrine disrupting activity with *ex vivo* whole rat embryo culture

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Standard animal tests to evaluate the safety of pesticides can be prohibitively expensive. However, rat post-implantation whole embryo culture (WEC) is a promising alternative test to assess developmental toxicity. In this study, we suggest proposed an integrated approach to screen for reproductive, developmental, and endocrine disrupting activities using *ex vivo* whole rat embryo culture. Specifically, our proposed approach evaluates adverse outcome pathways (AOPs), chemical structure activity relationships (SARs), morphological scores, receptor activities, and immunohistochemistry in rat WEC. We also employed WEC to assess endocrine-disrupting activity induced by environmental chemicals, which to the best of our knowledge, had not been done before. All experiment results in this study were comparable with those of OECD test guidelines for reproductive toxicity (443, 415, 416), developmental toxicity (414, 421, 422) and endocrine disrupting activity (426, 440, 441). Results revealed that, during rat embryo development, 17 $\beta$ -estradiol, triiodothyronine, triadimefon, penconazole, propiconazole did not significant affect the yolk sac circulatory system; allantois; flexion; the heart caudal neural tube; the hindbrain, midbrain, or forebrain; the otic system; the optic system; the olfactory system; the maxillary process; the forelimb or hind limb; yolk sac diameter; crown-rump length; head length; or developmental score. Immunohistochemistry revealed that 17 $\beta$ -estradiol (which was used as a positive control) positively affected its receptor expressions. These three triazoles induced ER $\alpha$  and ER $\beta$  expression in WEC. This result illustrates the triazole mode of action, in which triazole compounds disrupt steroid hormone synthesis. Finally, the research performed herein confirmed that our proposed method is both effective and fast, requiring only 10.5 days to detect (1) hormone receptors, such as androgen, estrogen, and thyroid receptors, (2) aromatase receptors and aromatase activity, and (3) developmental and reproductive toxicity. Therefore, our integrated approach to screening for reproductive, developmental and endocrine disrupting has the potential to benefit pesticide testing and assessment applications.

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**Presentation:** Oral

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## The Berlin-Brandenburg Research platform BB3R – Research and graduate education since 2014

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The Berlin-Brandenburg Research Platform BB3R, founded in 2014 at Freie Universität Berlin, bundles the 3R-related competencies of the Berlin-Brandenburg region and promotes systematic research in this area. The integrated research training program is the first in the world to offer structured qualification of young researchers in the field of 3Rs fostering the implementation of respective approaches into preclinical research. The project received start-up funding from the Federal Ministry of Education and Research (BMBF) and operates under the umbrella of the Dahlem Research School (DRS).

11 founding members conduct research in the fields of skin disease models, immunology, human-on-a-chip, nanotoxicology, *in-silico* drug analysis and drug design (reduction/replacement). For non-replaceable animal experiments, a strain-specific pain therapy in the experimental animal (refinement) is developed and stress is investigated by multiple experiments also with the aim of reduction. Three assistant professorships expanded the circle of experienced scientists from the beginning. Nine designated scientists strengthen the consortium as associated members. The first graduates now hold positions in national and international institutions like Federal Office of Consumer Protection and Food Safety, Federal Institute for Risk Assessment, National Institutes of Health, Industry, as well as holding professorships at the Universities of Bonn and Vancouver.

In addition to the research project, the graduate program also includes, doctoral symposia, seminars and annual spring schools with special emphasis on imparting knowledge of all 3Rs. General skills are taught in courses at the superordinate DRS. External doctoral students can be affiliated to the Research Training



Group, provided that they work in one of the 3R areas and meet the quality requirements of DRS.

The ideas have been taken up resulting in the foundation of Charité 3R. Since 2020, 3Rs also receive specific funding from the Berlin House of Representatives.

**Presentation:** Oral

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## The EUSAAT initiative to establish a European network of 3Rs centers

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The purpose of this network is to bring European 3R centres and societies together to share best practices, enhance communication, support the exchange of information and prepare the ground for common initiatives.

After an initial meeting of representatives of 3Rs centres and societies at the EUSAAT conference in September 2018 in Linz (Austria), the first follow-up meeting was hosted by Freie Universität Berlin and took place in March 2019.

Major common aims were identified: to further advance the 3Rs, to help implement the aims of Directive 2010/63/EU locally, and to reach out and connect with scientists in basic research. The network could be used as a platform to exchange experiences on a variety of topics, for example: how the various 3R centres and societies have been built up, how they organize events, how they secure funding, which teaching strategies and resources they use to implement 3Rs in education et cetera.

During the discussions it became clear that the diversity of the members could be the strength of the network, since they cover many different topics and have experts on Refinement, Reduction and Replacement of animal experiments.

Topics with an urgent need were identified and working groups for these topics have been defined. The progress of these working groups was presented at the third meeting during the FELASA conference in June 2019. In October 2019 at the fourth meeting at the EUSAAT2019 conference in Linz future initiatives were decided and agreed on.

So far, members from over 25 countries have participated and the network is growing.

The network is a completely independent, open and free community, which is very much dependent upon initiatives of its protagonists and personal efforts. It is based upon a bottom-up approach, and every 3R centre or society is welcome to join.

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**Presentation:** Oral

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## Promotion of the 3Rs consensus formation in China through transformation between academia and industry

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Implementation of 3Rs and its alternative methods requires the consensus and harmonized action among multilateral institutions including academia, regulation and industry. However, in China this situation lags far behind, such as lack of a close circle to coordinate and promote each other, absence of the rules and regulations to outspread the transformation and application of 3Rs and its alternatives.

The concept of alternative to animal testing has been widely used in safety and efficacy testing in the area of food, cosmetics, ecology and nanomaterials which contributes to several important scientific findings. Both the School of Public Health in Shanghai Jiao Tong University and the CCARE (Consensus Center of Alternatives Research & Evaluation) of Shanghai Society of Toxicology have made a lot of efforts most recently in the translation of academia to industry on 3Rs and its alternatives based on the huge academic and industrial influence on medicine, biotechnology and toxicology.

We have hosted 8 international conference of alternatives and organized the 3Rs continuing training courses for the industry with more than 1,000 attendees (Cheng et al., 2017). It is important for Chinese companies to have sufficient personnel and technical capabilities to conduct non-animal testing in consistent with the regulations change in the future. We firstly opened the translational toxicology courses for graduate student in China university 3 years ago. We make a long term plan to promote the scientific public on 3Rs and non-animal testing.

Through unremitting efforts, we have now reached a preliminary consensus among academia, regulation and industry in China and promoted the international cooperation around the world.

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**Presentation:** Oral



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## Asian activities for alternatives to animal experiments

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In 2016, the Japanese Society for Alternatives to Animal Experiments (JSAAE) launched the first Asian Congress on Alternatives to Animal Experiments to promote throughout Asia the Three Rs—Replacement, Reduction, and Refinement—as guiding principles for a more ethical use of animals in scientific testing. Following the First Congress in Karatsu, Japan, during November 2016 and the Second Congress in Guangzhou, China, during October 2018, a Third Congress is now being planned for Korea during 2021. And to promote closer collaboration between our Asian colleagues, we are also now exploring the possibility of organizing for 2020 an Asian Consortium, which will provide funding and human resources in support of future Congresses in Asian countries.

Although our Asian colleagues are deeply involved in research and promotion of the Three Rs, countries in the EU and the USA are still the leading advocates for these principles, and we look forward to strong support for the Asian Consortium from researchers around the world.

**Presentation:** Oral

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## Systematic reviews to validate alternatives to specific animal models

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Animal experiments are legally required in drug development. When replacing an established animal model by an alternative, validation is necessary. Systematic Reviews (SRs) can aid. We here focus on establishing objective values of the probabilistic translational success rates of recognized animal models. These values can be used as a reference in external validation of alternatives. The presentation will comprise the results from 2 unrelated SRs.

Our first, recently published, SR shows that studies of probabilistic translational success rates found different values, which range from 0 to 100% (Leenaars et al., 2019). This range is based on 121 included references, with various units of measurement to determine translational success: compound or in-

tervention ( $k = 104$ ), study/experiment ( $k = 10$ ), and symptom or event ( $k = 7$ ). Probabilistic translational success rates were calculated following several definitions, comprising percentages below 2-fold error, percentages accurately predicted, predictive values, correlation/regression ( $r^2$ ) and percentage overlap of the 95% confidence intervals in meta-analyses. Translational success rates did not clearly relate to species, type of calculation, publication date or broadly defined research field. However, the included studies generally had a high risk of bias.

Our second review is still in progress; analyses will be finished at the time of the conference. This review compares the experimental design in animal and human trials based on a case study; methotrexate to treat rheumatoid arthritis. The search retrieved 8217 references. After screening, 695 papers were included in the SR. Approximately 25% of the included papers is on animal studies, the remainder is on human studies. The results will be discussed in light of translational success and the development of alternatives to animal studies.

We encourage SRs for probabilistic evaluation of the translational success of *in vivo* models and *in vitro* alternatives over mechanistic evaluation.

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**Presentation:** Oral

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## Towards an automated facial expression analysis in mice using deep learning

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The Mouse Grimace Scale (MGS), a coding system for facial expression analysis of pain in mice, has been widely accepted as an important welfare indicator among experts in laboratory animal science over the last years (Campos-Luna et al., 2019). However, since this method is usually manually applied by human ob-

servers, it can be assumed that the MGS scores are subjective to a certain extent. Therefore, the reliability of this method is frequently questioned. Moreover, if facial expressions of mice are analyzed in real time by human observers standing right in front of the cage and scoring the mice, the presence of the humans can influence their behavior. Mice are prey animals and often hide signs of negative affective states when humans are present (Stasiak et al., 2003). To circumvent these problems, we aimed to develop a facial expression recognition software for mice (Andresen et al., 2019). We utilized a dataset including images of adult male and female C57BL/6JRj mice, that were either anesthetized with isoflurane or ketamine/xylazine, castrated (under isoflurane, meloxicam, lidocaine/prilocaine) or untreated. The image dataset was divided into two categories, i.e., post-surgical/anesthetic effects and no post-surgical/anesthetic effects, and a binary classifier was trained to differentiate between the categories. We used three convolutional neural network (CNN) architectures (two pre-trained state of the art deep CNN: ResNet50 and InceptionV3; one CNN of our own design without pre-training) and achieved an accuracy of up to 99%, which can keep up with the human performance. Moreover, Deep Taylor decomposition – a feature visualization technique – indicated that the decision of the network was indeed mainly based on image areas depicting the mouse faces (Andresen et al., 2019). Our first steps towards a fully automated facial expression recognition software provide a significant contribution to refining pain and stress assessment in laboratory mice.

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**Presentation:** Oral

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## Implementing cup and tunnel handling in a (large) pharmaceutical rodent facility

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The standard way of catching, lifting and handling mice has for decades been by grasping the animal by the tail, lifting it by the tail and generally holding the mouse by the tail always, because this was considered the only effective and safe way of handling mice.

In 2010 the first article on cup- and tunnel handling was published (Hurst et al., 2010). This and following articles (peer-reviewed) since then demonstrated that catching, lifting and handling mice by either cup- or tunnel handling is less stressful and creates more trust for the mice.

Already in 2015, Novo Nordisk began to look into tunnel- and cup handling, however, without the correct education and instruction in these methods, we had to put it aside for some time.

In late 2017 a decision was taken at management level, that cup and tunnel handling methods should be implemented, beginning in 2018.

Emphasis was on the following:

- Employees had to change their way of thinking, as well as their way of acting.
- The employees had to be educated in both the theory as well as the practical aspect of the new methods before beginning the implementation.
- Time had to be dedicated to training and evaluating.
- Equipment (tunnels) had to be in place in each cage.

With a dedicated plan and intensive follow-up, a full implementation has been reached, as well as a mind-set change amongst the animal caretakers.

Tunnel and cup handling of mice can be applied in any animal facility around the world. However, training of personnel and management endorsement is essential and necessary, and if tunnel handling is desired, tunnels must be provided. Other than this, it is neither costly, more time consuming nor difficult to do.

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**Presentation:** Oral



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## Critical incident reporting system in laboratory animal science – CIRS-LAS

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CIRS-LAS (Critical Incident Reporting System in Laboratory Animal Science) is the world's first online available database for critical incidents in the entire range of laboratory animal science (Bischoff et al., 2018), based on similar obligatory systems in the human medicine.

From today's perspective and considering the current state of science, use of animals in experimental purposes cannot be completely dispensed. A large number of scientific articles based on animal experimental studies are published daily. But negative experiences gained from these experiments get lost or are not referred in publications.

On <https://www.cirs-las.de/> any person involved in laboratory animal science can send an anonymous report about critical incidents to share their experiences or to give possible suggestions for improvement. Additionally, registered persons are allowed to read and comment other reports. Currently, more than 130 people from Europe are already registered users of the CIRS-LAS portal and more than 50 critical incidents have already been entered. The objective of CIRS-LAS is an open dialogue about failures which can help to avoid them in the future. CIRS-LAS plays an exemplary pioneering role in the implementation of the 3R principles: reduce the number of laboratory animals (reduction) and improve animal safety (refinement). CIRS-LAS provides an online platform for an open-minded and active failure management and supports exchange of negative experiences and possible solutions to improve animal welfare (Enkelmann and Bischoff, 2020). Transparency in lab animal science starts now – it's time to join CIRS-LAS.de!

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**Presentation:** Oral

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## Retrofitting an *in vitro* TOX21 high throughput screening assay for P53 activation with metabolic capability: Comparing results from human and rat liver microsome preparations

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The Tox21 Program has screened a 10,000 compound library in over 80 cell-based quantitative high throughput screening (qHTS) assays. These assays employ 1536-well plates on a robotic platform; all assays are homogeneous (“add, mix, measure”) with no aspiration steps due to the small assay volume per well (~5 µL). In standard *in vitro* assays used routinely to characterize genotoxicity (e.g., bacterial mutation or mammalian cell micronucleus assays), induced rat liver S9 fraction plus co-factors is employed as a source of Phase I enzymes to mimic *in vivo* metabolism of the compound under study. However, S9 enzymes are toxic to cells after a few hours of exposure. Therefore, in these standard assays, medium plus S9 mix is removed after ~4 hours and replaced with fresh medium without S9. Due to the inability to remove S9 mix by aspiration, Tox21 cell-based assays have had limited or no metabolic capability. This lack of biotransformation capability is reflected in the inability of the Tox21 DNA damage assays to detect most compounds that require activation to a DNA reactive metabolite (e.g., pyrene). This limitation also risks mischaracterization of compounds that are detoxified by liver metabolic enzymes. Successfully retrofitting Tox21 assays with metabolic capability would achieve a key goal in the new Tox21 strategic plan of providing data with enhanced physiological relevance. We therefore investigated whether low, non-toxic concentrations of human or induced rat liver microsome preparations could provide effective metabolic capability to the cell-based p53 reporter gene assay, which measures activation of p53, a gene that responds to DNA damage. Results showed both microsome preparations were biologically active, but differences were seen in the number and identity of chemicals that were metabolized. Interpretation of these data and applications for use of microsome preparations in high throughput screening assays will be explored.

**Presentation:** Oral

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## Replacing foetal bovine serum: A piece of cake?

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FBS is still being applied as the universal medium supplement to grow and maintain cells and tissues. But, the use of FBS presents five significant issues:

1. the degree of suffering experienced by the calf during blood collection (van der Valk et al., 2004);
2. inappropriate cellular growth profiles and physiological responses of cells cultivated with medium containing FBS;
3. FBS contamination with viruses, prions, etc.;
4. the large variability of FBS such that it is very difficult to even ensure consistent and well-controlled *in vitro* cell culture between batches;
5. the fraud-problem (Gstraunthaler et al., 2014).

To answer to these issues, the use of FBS in particular, but also the use of other animal-derived products in cell, tissue and organ culture and related experimental techniques should be avoided wherever possible. There are several strategies to avoid the use of FBS. Human platelet lysates (HPLs) can be a valuable alternative to FBS as cell culture supplement. In addition, there is large interest in chemically-defined media (van der Valk et al., 2018). As HPLs are undefined but work for most cell types, chemically-defined media, on the other hand, are cell type-specific. To facilitate the search for serum-free media, the fcs-free.org database was established. This database provides an overview of commercially available serum-free media for cell and tissue culture, as well as medium formulations for specific cell types obtained from scientific literature. Furthermore, the website serves as a platform to exchange information on the quality and applicability of each product. Not for every cell type is yet a serum-free medium available. Strategies will be discussed to develop a serum-free medium for a specific cell type and to adapt cells to the new medium (van der Valk et al., 2010).

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**Presentation:** Oral

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## Animal use for science in Europe

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With more than 80% of national competences being centralized by the European Commission (EC) on behalf of the EU member states, Brussels is the place to be when it comes to interact with the policy makers (e.g., Members of the European Parliament (MEP)). EC transparency register has collected in February 2019 almost 12,000 entries claiming lobbying activities in the Belgian capital. Animal Welfare and 3Rs are not always a central topic being discussed when Brexit, Greek debt, migration crisis, terrorism attacks, glyphosate re-authorization are also steering the attention. In other words, the 3Rs topic competes with all these major issues that policy makers deal with every day. Therefore, it is essential to map the policy environment in order to better communicate 3Rs and be efficient with policy makers by 1) identifying the actors, 2) the institution we are dealing with 3) the pressure participants 4) linking 3Rs with the context and events (i.e., political agenda) 5) providing appropriate evidence that fits policy concerns at hands and last but not least 6) building trust on the long term. For the latter, an initiative was designed to provide inter-sector information exchange for future actions is the “MEP – 3Rs scientists pairing scheme” initiated in 2015 by CAAT-Europe at the European Parliament. It was the opportunity to identify some of the obstacles when it comes to integrate scientific advice/expertise with values represented by elected policy makers.

**Presentation:** Oral

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## Setting the scene for a new paradigm for risk assessment: Evolution versus revolution

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Can innovative approaches in chemical safety assessment, focusing on the human and avoiding animal testing, change the regulatory landscape? First, the current evolutionary scenario makes changes one by one within the current regulatory frame-



works. Alternatively, a revolutionary approach would start from building an *in silico* virtual human physiological model for chemical safety assessment. The model would provide the integrated safety assessment for the tested chemical, being fed by data from testing a chemical in dedicated *in vitro* assays for critical rate-limiting steps in the physiological regulatory network. Individual assays will still need characterization in terms of their biological mechanism(s), their reliability/reproducibility, and their chemical applicability domain. However, predictivity of individual assays for toxicity in the intact organism is irrelevant in this approach. Rather, confidence in sufficient coverage of the biology by the combined battery of fit-for-purpose assays, reflecting the rate-limiting key events in the network, should suffice for considering a testing strategy valid. This system combines existing biological, chemical and toxicological knowledge and may well require reformulation of practical requirements for regulatory chemical safety assessment. Safety assessment through the virtual human has to prove itself in actual practice, based on experience in the human. As scientific reliability of such a system is growing, it is important to simultaneously raise confidence of all stakeholders, including the general public. Of course, the stakes are high when it comes to human health protection. Although major hurdles still need to be taken, accelerating advances in machine learning and *in silico* modelling of human development, physiology and disease suggest that the virtual human and its application in chemical safety assessment may be closer at hand than anticipated heretofore.

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**Presentation:** Oral

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### Mind the gap: Improving literature search skills to access the most relevant scientific and tech knowledge

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About 20 million scientists in the world publish roughly five papers per minute, with an increasing trend of 8-9% per year. Such

an overload of information is likely to cause stress and frustration to most researchers, giving the erroneous impression that there might be no much left to research on. However, not only the 2.5 million scientific publications per year are a proof of the contrary, but they may also turn to be a great source of inspiration. Indeed, underneath this huge amount of information, knowledge and technology gaps are hiding and literature search becomes a treasure hunt.

Most of the researchers likely use their favorite web search engine and begin an iterative, often endless, typing. Are there better keywords that could be used? How to search for something that perhaps does not exist? And how to assess the relevance of the retrieved information? Here, we will provide you with clues and a series of critical steps to identify gaps in specific research fields of interest. The systematic search approach allows acknowledgment of relevant literature and provides keys to answer open questions, while saving time and money by avoiding to reinvent the wheel. Finally, and most important, our proposed suggestions will allow to gain insights and seed ideas for future grant proposals and/or patent submissions, thus opening new avenues to research innovation.

**Presentation:** Oral

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### Biology-inspired microphysiological system approaches to solve the prediction dilemma of substance testing

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Both the reported shortcomings of animal studies regarding their predictivity for humans as well as the desire to reduce the dependence on animal testing in drug development inspired the hope for microphysiological systems or organ-on-a-chip.

As with any new technology, it became evident, that it was necessary to channel the initial hype into realistic expectations to avoid subsequent delusions. CAAT was instrumental in this process by organizing a workshop in the framework of their transatlantic think tank for toxicology. This workshop, held in 2015, brought together stakeholders from the pharmaceutical industry, academia, technology providers and regulators. The group exchanged the state of the development of the technology and developed a road-map for the implementation of microphysiological systems within the drug development pipeline (Marx et al., 2016). The workshop triggered a series of interactions and collaborations between pharmaceutical companies and technology providers. The success of the workshop is evidenced by a follow-up event held in 2019.



The presentation will summarize the results of the initial workshop and discuss the level of implementation in the light of the recent developments.

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**Presentation:** Oral

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### Case studies of industrial adoption using microphysiological systems

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The Pharmaceutical industry has implemented a broad array of *in vitro* systems to support preclinical evaluation of new drug candidates. Microphysiological Systems (MPS) are considered to further improve prediction of safety and efficacy of new drug candidates prior to their use in humans. MPS-based assays are increasingly becoming part of the internal decision-making processes within Pharma and this session aims to present case studies of industrial adoption while discussing their impact on the 3Rs.

**Presentation:** Oral

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### Exposure of lung cell models to complete unfiltered and filtered exhaust from gasoline/diesel cars and comparison with findings in humans

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Pollution from vehicles is a major environmental problem due to the various components in the exhaust gases emitted into the atmosphere. Many studies clearly show the link between air pollution and a wide variety of human respiratory and systemic health complications (Riediker et al., 2019). In addition, a link has been

shown between the increase in allergic diseases such as asthma and increased exposure to air and traffic (Murrison et al., 2019). As combustion engines are one of the most important sources of air pollution, its emission regulations have become increasingly stringent in recent decades. Consequently, new strategies have been and are developed to improve engine combustion and efficiency and exhaust after-treatment. However, which biological mechanisms are linked to which emission components are not yet clear.

The aim of this presentation is to provide an overview of the current state of research and clinical aspects in this field, with a focus on the available *in vitro* lung models for studying pulmonary toxicity, inflammation, and immune effects. Data are presented that show that the combination of an air-liquid interface exposure system and 3D lung-cell culture model provides a suitable tool for rapid and reliable investigations of complete exhaust toxicity as well as the effects of particulate fraction, i.e., filtered exhaust (Bisig et al., 2018; Steiner et al., 2016). Such studies provide important results for new and improved emission technologies in the early stages of product development and will also help to correlate the source of exposure with the adverse immune effects observed in humans.

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**Presentation:** Oral

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### New ideas for systemic toxicity: Outcome of an EPAA blue sky workshop

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EPAA held a blue sky workshop more than ten years ago to brainstorm about how new technologies in biotechnology, chemistry and computation could be applied to the problem of



predicting repeated-dose toxicity. Many of the ideas from that workshop led to the SEURAT program, which developed research tools that could support read across and ab initio approaches. Despite progress, there is still no agreed upon approach for systemic toxicity replacement, and little uptake into regulatory processes. EPAA held a second blue-sky workshop in Fall 2019 to consider whether new technologies should be incorporated into a replacement strategy, and to take stock of whether current and foreseen replacement approaches will meet regulatory needs. The participants included scientists from academia, industry and government, including regulators. The participants agreed that we are on the right track using read-across workflows that build upon historical toxicology testing data supported by biotechnology and computation. It was also clear that we will need a much deeper understanding of systems biology and how perturbations lead to adverse outcomes. Specific recommendations included development of comprehensive ontology of modes of toxicity, global cataloging of biological responses to toxicants through ‘omics, better understanding of 3D protein structure and how small molecules interact with macromolecules, and higher order biological and computational models (like organoids and virtual organs) that allow interrogation of the link between initial interaction at the molecular level and adverse outcome. Case studies will be important in helping regulators understand how alternative approaches are used. The participants also urged data to be shared publicly to accelerate progress. The full recommendations of the workshop will be presented at the conference.

**Presentation:** Oral

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## Cheminformatics and gene expression data: Linking chemical-biological interaction to outcome

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Adverse outcomes *in vivo* are the culmination of a series of steps that begin with the interaction of an exogenous chemical and its molecular target. Organizing toxicity information based on chemical structure and mode of action (usually at the molecular level) provides a foundation for selecting higher order models and assays to fill in the rest of an adverse outcome pathway (AOP). We are developing ontologies for several modes of toxicity, beginning with developmental toxicity and hepatotoxicity. Gene expression data are being used to verify similar actions of chemicals and to help identify pathways by which chemicals act. This, in turn, informs the selection of appropriate models for further investigating the mode of action leading to a particular outcome. The initial applications of these approaches are in support

of read-across, but they have the potential to support assessments for chemicals that have no analogs.

**Presentation:** Oral

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## Strategizing to optimize the development and use of animal-free antibodies in the U.S.

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Building on the Scientific Advisory Committee of the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) findings that the use of animal-free recombinant antibodies would improve scientific reproducibility, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the PETA International Science Consortium Ltd. convened the expert meeting “Developing strategies to increase the use of recombinant antibodies” in December 2019. Meeting participants identified parameters that animal-free recombinant antibodies would need to meet in order to achieve broader use throughout the scientific community and challenges that have hindered their adoption despite their scientific benefits. With this background, participants outlined a pathway that will foster a large-scale transition to animal-free recombinant antibodies, concentrating on the largest antibody market – antibodies produced for basic research. Meeting participants identified promising activities to increase awareness about the capabilities of animal-free recombinant antibodies and currently available resources and to increase funding and infrastructure for their development and validation. Committees on funding, partnerships, validation, and education and awareness were established to carry out these activities. The objective of this presentation is to provide an overview of barriers to the use of animal-free recombinant antibodies in the United States and how the committees are addressing these barriers.

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**Presentation:** Oral

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## EU-NETVAL Thyroid Validation Study: Chemical selection strategy

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As a follow up on the European framework for Endocrine Disruptors (EC, 2018), EURL ECVAM is coordinating the validation of 18 thyroid-targeted test methods (OECD, 2014). The aim is to assess the potential of chemicals to disrupt the thyroid hormone (TH) axis, including various modes of action. Fourteen laboratories from the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL), are participating in this two-part validation study:

Part 1, currently ongoing, aims to define the methods by standard operating procedures (SOPs) with assessment of reproducibility and reliability. Known positive/negative control and reference chemicals, respective for each method (OECD, 2018) are being used, identified previously during test method research and development.

Part 2 aims to assess the overall relevance of the test methods, using 30 validation set chemicals, selected on the basis of known modes of action and available *in vivo* study data screened using all 18 methods.

A challenge for the success of this validation study is the appropriate selection of relevant validation set chemicals to be tested, which should cover the range of expected response from the various modes of action. In support of the chemical selection for

Part 2, an expert meeting was organized in November 2019. Prior to this meeting, experts were surveyed to propose potential validation set chemicals (OECD, 2018) with known activity in at least one of the methods/mechanisms of action: 87 chemicals were initially suggested. During the meeting, these initial proposals were reduced to a short list of 51 chemicals, including negative control chemicals, which in the collective opinion best cover the methods and their modes of action. The shortlist will undergo further refinement over the coming months to yield 30 validation set chemicals.

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- OECD (2018). Guidance Document on Good *In Vitro* Method Practices (GIVIMP). *Series on Testing and Assessment, No. 286*. OECD, Paris.

**Presentation:** Oral

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## AOPs for endocrine disruptors

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The regulatory requirement for identifying chemicals as endocrine disruptors implicitly or explicitly requires data identifying an adverse effect in animals and mechanistic data indicating an endocrine mode of action. A variety of guideline test methods are available for evaluating the endocrine hazard of chemicals, but few include both of these types of data and it is general anticipated that information generated by different test methods will be needed in order to reach a conclusion. This presentation will provide examples on how endocrine data are integrated and discuss the strengths and weaknesses of the approach.

**Presentation:** Oral



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## The impact of the CAAT publication series on ALTEX

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The recognition of the journal ALTEX (<https://www.altex.org>) as a popular venue to publish high-quality work relevant to the 3Rs took off ten years ago and has continued to grow since then as evidenced by the journal's impact factor, submission numbers, number of manuscripts published per year, breadth of subjects covered, and number and diversity of contributors. A large portion of this development is attributable to publications coming from CAAT and CAAT-Europe during this time, i.e., the Food for Thought ... series (started in 2007), t<sup>4</sup> reports and t<sup>4</sup> workshop reports (started in 2008), the BenchMarks series (started 2018), and numerous original publications. Food for Thought ... are opinion articles that challenge the status quo and envision scientific strategies for new developments. t<sup>4</sup> workshop reports bring interdisciplinary leaders together to cooperate on formulating expert opinions and to set out a path towards a common goal, and the BenchMarks articles aim to improve the quality of science in the field by highlighting technical challenges and shortcomings. Many of the authors and reviewers ALTEX has worked with through these unique and highly cited formats continue to return as contributors or to recommend ALTEX to others. Regular updates on CAAT activities, titled CAATfeed, inspired the Corners section where participating institutions can relate their 3R relevant news to the community. The fruitful collaboration between CAAT/CAAT-Europe and ALTEX has enabled ALTEX to grow and mature as a journal and thereby to promote the field of new approach methods by showcasing high-level human-relevant science.

**Presentation:** Oral

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## Mimicking human lung tumour complexity in 3D *in vitro* tumoroids cultured at the air-liquid interface for predicting the efficacy of inhaled anti-cancer drugs in NSCLC patients

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With 353,000 deaths every year, lung cancer is the leading cause of cancer-related deaths worldwide. Non-Small-Cell Lung Cancer (NSCLC) is its most common form (incidence, ~85%), and adenocarcinoma is the most frequent NSCLC subtype (~50%).

Lung cancer has no cure. Seven out of eight patients die within five years from diagnosis. Of these, 80% die within one year. Thus, there is a critical need of developing more effective treatments against such disease. Inhalation has demonstrated several advantages over other drug-administration routes in NSCLC treatment. Due to oversimplification, technical limitations and interspecies differences, however, currently available preclinical (*in vitro*/animal) models do not support the development of inhaled chemotherapeutics.

Here, we present our advances in the development of human-relevant, tissue-mimetic *in vitro* models that closely mimic patient responses and are compatible with testing inhalable anti-NSCLC drugs (Movia et al., 2018, 2019). Our *in vitro* models are formed by growing, for the first time, 3D tumoroids of human adenocarcinoma (A549) cells in the presence (co-culture) or absence (mono-culture) of human fibroblasts (MRC-5 cell line) at the Air-Liquid Interface (ALI). The *in vitro* models developed were tested for their response to four benchmarking chemotherapeutics. Results demonstrated clinically relevant MultiDrug Resistance (MDR). The signalling pathways of the culture MDR, in fact, reproduced those found in NSCLC patients. Notably, such pathways were influenced by the cancer cell-fibroblast cross-talk, which was mediated *in vitro* through TGF- $\beta$ 1 release and subsequent activation of the PI3K/AKT/mTOR pathway, as per *in vivo* conditions. Finally, compatibility with testing drugs administered as a liquid aerosol by a clinical nebulizer was demonstrated.

In conclusion, our data demonstrate that our *in vitro* models provide a first alternative to animal studies for the efficacy screening of inhaled chemotherapeutics, thus opening new research avenues in the development of more effective anti-NSCLC drugs.

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**Presentation:** Oral

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## Moderated panel discussion: Is it time to say “Bye bye Buehler”?

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The goal of this workshop is to discuss opportunities and challenges to replace the Buehler test that has been used for decades with new approach methods (NAMs) for skin sensitization. During this workshop, the audience will hear presentations on the current status of NAMs for skin sensitization as well as how these are being used in different regions of the world. During this moderated panel discussion the audience as well as the presenters of the workshop are invited to discuss if it is possible to replace the Buehler test. The aim of this panel discussion is to identify the most important drivers and barriers that influence the acceptance and use of the NAMs. A better understanding of these aspects can be used to build confidence in NAMs for skin sensitization and may ultimately make the Buehler test redundant.

**Presentation:** Oral

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## Eye-on-chips: Next-generation microphysiological *in vitro* models for ophthalmology research and ocular toxicology

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The devastating effects and incurable nature of hereditary and sporadic retinal diseases such as Stargardt disease, age-related macular degeneration or retinitis pigmentosa urgently require the development of new therapeutic strategies. Additionally, a high prevalence of retinal toxicities is becoming more and more an issue of novel targeted therapeutic agents. Ophthalmologic drug development, to date, largely relies on animal models, which often do not provide results that are translatable to human patients. Hence, the establishment of sophisticated human tis-

sue-based *in vitro* models is of utmost importance. The discovery of self-forming retinal organoids (ROs) derived from human induced pluripotent stem cells (hiPSCs) is a promising approach to model the complex stratified retinal tissue. Yet, ROs lack vascularization and cannot recapitulate the important physiological interactions of matured photoreceptors and the retinal pigment epithelium (RPE). One promising approach to recapitulate human biology *in vitro* is by combining hiPSC technology with microfluidic devices tailored to create microphysiological environments and recapitulate 3D tissue structure and function. Such organ-on-a-chip platforms or microphysiological systems combine human genetic background, *in vivo*-like tissue structure, physiological functionality, and “vasculature-like” perfusion.

Using microfabrication techniques, we have developed the retina-on-chip (RoC), a novel microphysiological model of the human retina integrating more than seven different essential retinal cell types derived from hiPSCs. It provides vasculature-like perfusion and enables, for the first time, the recapitulation of the interaction of mature photoreceptor segments with RPE *in vitro*. We show that this interaction enhances the formation of outer segment-like structures and the establishment of *in vivo*-like physiological processes such as outer segment phagocytosis and calcium dynamics. In addition, we demonstrate the applicability of the RoC for drug testing, by reproducing the retinopathic side-effects of multiple compounds. The developed RoC has the potential to promote drug development and provide new insights into the underlying pathology of retinal diseases.

**Presentation:** Oral

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## Communicating animal research: A PLOS ONE perspective

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Scientific advance relies on transparency, rigour and reproducibility. The use of animals in scientific research is central to biomedical disciplines and brings with it the responsibility to maximize the knowledge gained from *in vivo* research. Improving the quality of reporting of published studies and sharing of the data are key in working towards reproducibility in research findings. Since its inception, the open access multi-disciplinary journal PLOS ONE has striven to publish work that is reproducible, as well as rigorously and ethically conducted. We will be discussing the policies and initiatives the journal has driven around research involving animals, and our commitment to promoting reproducibility and transparency in the biomedical fields.

**Presentation:** Oral



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## From research to innovation: Business in life sciences

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We become scientists because we all want the world to be a better and healthy place. In addition, science and research is the most energizing and stimulating experience your brain can live. As researchers, we live everyday asking questions with no answers. The adrenaline pumps up when you start generating bits and pieces of results and having some hints about a plausible, but a still very preliminary, answer. Then we shift to depression when we realize months of experiments were just on the wrong direction. However, we are quickly back to our desk with tons of articles and a new experimental plan to crash the bench. Finally you publish your scientific results, your endorphins and serotonin levels are at the top. You party all night and the next morning you start again. Will this article really make the world a better place? You spread your idea into the world, but you still need someone able to understand that idea and transform it into something real that people can benefit of. And business is a very key piece in this process, named innovation. This is why some of us go from research to business to keep pushing science to make our world a better place.

**Presentation:** Oral

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## Reproducibility crisis in preclinical research

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Several studies have demonstrated that quality and reproducibility represent major issues in the preclinical development of the translational pipeline, particularly in the process of drug development. Up to 50% of the investment at the preclinical level is mostly wasted. The cause of this lack of reproducibility due to elements that are actionable and the result of this situation leads to a high attrition rate in the drug development pipeline, particularly in clinical trials at stage II where the novel molecule confronts for the first time the clinical situation for which the drug was designed for. Challenges and opportunities for tackling this challenge will be discussed during the session.

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## What is the analysis of biomedical research literature teaching us about the use of non-animal models?

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Originality is the seed of innovation. Original ideas, questions and approaches are rewarded in life science with high-impact factor publications. High-impact factor publications give us visibility and the possibility to apply for new funding and to ask more original questions to start again. This is an established reward system in life science. Every researcher is trying to be as much as original is possible to keep up in this system. One of the key factor to be truly original is studying thoroughly the state of art, for not reinventing the wheel and to add real value. However, after screening more than 250,000 publications, my team and I think that a large number of researchers are not good as they should in studying the state of the art. And most importantly, we start believing that originality is hampering non-animal models research.

Our literature review analysis in four fields of biomedical research has revealed most of the time poor originality and many limitations in our way of planning, developing, characterizing and using non-animal models. However, thanks to this enormous project funded by the European Commission, we are now able to propose some working solutions to improve the originality of our research and to offer more relevant non-animal models for their use. And one of them is collaboration.

**Presentation:** Oral

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## Barriers and challenges facing the replacement of animal-derived antibodies (ADAs)

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Animal Friendly replacement methods for Animal Derived Antibody (ADA) production have been available 20+ years. Yet despite their unequivocal scientific advantages over vintage animal immunisation-based techniques, uptake has been poor. ADAs continues to be relied upon in every scientific discipline for immune-analysis, including safety and batch testing, health care management, therapeutics, R&D and even cosmetics efficacy testing, where animal procedures are supposedly banned in



the EU but where antibody production as a scientific procedure is overlooked (Gray et al., 2016a,b). Widespread resignation toward antibody quality, originating from the inherent shortcomings of ADAs is incorrectly perceived as an unavoidable hurdle. The reasons for poor uptake of replacement methods are not due to lack of replacement options, or molecular properties of Animal Friendly Affinity-reagents (AFAs) themselves, since these are well developed. Instead a tangled web of issues including a propensity toward existing methods, significant scientific misconceptions regarding their replacements; perceived economic and contractual constraints; lack of guidance regarding availability; limited access to genuine catalogue AFAs; focus on the more lucrative therapeutics market preventing its widespread commercial diffusion; and a lack of focus on ADAs as a scientific procedure and subsequent enforcement of Directive 2010/63/EU. Collectively, this means large scale generic project applications proposing the use of hundreds or thousands of animals for broad scale antibody generation are still authorized in EU member states. In addition, there is a resounding impact on the scientific community of using non-specific reagents in terms of cost, time and resources and society continues to suffer unquantifiable repercussions on diagnosis and health management (Bradbury et al., 2015). We are now facing an exciting new era, where these barriers and challenges are being confronted. AFABILITY, EURL-ECVAM and partners are collaborating to ensure uptake of replacement methods for ADA production is a global achievement (<https://publications.jrc.ec.europa.eu/repository/>; Groff et al., 2020)

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## Next generation risk assessment for consumer safety: What do we need from validation?

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The principles identified by the International Cooperation on Cosmetics Regulation for the use of new approach methodologies (NAMs) in Next Generation Risk Assessment (NGRA) include that risk assessments should be designed to prevent harm in consumers, be exposure-led and that any data generation should be tiered and iterative. Assessments should be based on robust and relevant methods and strategies (Dent et al., 2018).

An approach to the evaluation of NAMs for use in NGRA for both skin allergy and systemic effects will be discussed. In both cases, detailed information on levels of exposure to benchmark substances that can cause adverse effects in humans drive the evaluation process: either through (1) known levels of skin exposure to materials with documented evidence regarding the ability of that exposure to induce skin sensitisation; or (2) systemic exposure estimates (using physiologically-based kinetic modelling; Moxon et al., 2020) for materials with documented evidence regarding the ability of that exposure to induce systemic adverse effects.

In this context, two approaches to the evaluation of NAMs for use after an initial *in silico* NGRA tier will be described: (1) an approach for skin allergy using experimental data from DPRA, hCLAT, Keratinosens™ and U-Sens™ assays and a Bayesian probabilistic model to estimate human sensitiser potency (Reynolds et al., 2019) and (2) an approach for systemic effects using SafetyScreen44™, Cell Stress Panel (Hatherell et al., 2020) and BioSpyder Tempo-Seq, high throughput transcriptomic data.

This approach to evaluation of NAMs for use in NGRA seeks to establish a fit-for-purpose, robust and reproducible set of bioactivity assays that can be used to generate margins of safety for chemicals when combined with information on levels of consumer exposure.

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**Presentation:** Oral



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## Not in our back yards: OneHealth implications of the international primate trade

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The OneHealth initiative recognizes that the environment, domesticated animals, wildlife and public health are all part of the same, dynamic system. Using the OneHealth multidisciplinary framework, I will demonstrate that the intentional introduction of tens of thousands of monkeys into non-native ecosystems via “monkey farming” has the potential for significant disruptions in animal, human and environmental health. Hendry County, Florida is the epicenter for the commodification of macaques used in research in the United States. Here, thousands of primates are imported and stockpiled year-round in breeding and research facilities. The poor conditions that these primates are confined in are well known and documented. The importation companies cite Hendry County as an “ideal environment for nonhuman primates” noting the “tropical climate”, “agricultural mindset of the labor force” and the “knowledge base of environmental and regulatory agencies”. What is not acknowledged is that these monkey farming facilities are situated amongst commercial agricultural and livestock operations, residential farms, craft breweries and restaurants that rely on the rich agricultural lands in this poor, rural community. The macaques warehoused in Hendry County often arrive naturally infected with parasites, bacteria, mycobacteria, and viruses, many of which may present a public health concern. Crammed into poorly designed indoor/outdoor facilities the primates generate waste water contaminated with feces, urine, and blood. Additionally, macaques are attractive hosts for the mosquitos that can transmit vector-borne pathogens such as Zika, dengue and West Nile virus. The practice of monkey farming is a critical link in the use of primates for research. By drawing attention to the public health risks of warehousing large numbers of monkeys in places like Hendry County we may be able to break the supply chain and hasten the end of the use of primates in research.

**Presentation:** Oral

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## Recombinant antibody technology: Taking antibodies from bench to bedside

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To understand protein function, researchers require antibodies (Abs) against proteins to use as specific probes to assess protein function and location. Interest in Abs has been further spurred by their emergence as a major therapeutic breakthrough over the last decade for the treatment of cancer and other devastating diseases. Most monoclonal Abs (mAbs) are produced by hybridoma methods, which have changed little since their inception decades ago. The hybridoma technology suffers from several drawbacks: the difficult and labour-intensive development of a hybridoma, the immune bias of animal repertoires, and the controversial use of research animals. In recent years, research in protein engineering has given rise to “synthetic antibodies” which use man-made antigen-binding sites and thus circumvent the need for animals. We have developed synthetic antibodies that use a single human framework and limited chemical diversity restricted to regions of the antigen-binding site. Libraries of Ab fragments are displayed on phage particles. Each particle displays a unique Ab which encapsulates a vector that contains the encoding DNA. Phage pools containing billions of unique Abs are used in selections to isolate Abs that recognize specific antigens, and the sequence of each Ab is decoded by sequencing the linked DNA. The linked DNA sequences can be used to produce the Ab directly from bacteria or mammalian cells. These features of phage display technology obviate many of the limitations of hybridoma methods and are suited to high-throughput applications since the process relies on simple molecular biology techniques. Furthermore, synthetic Ab engineers are not limited by nature’s Ab formats; the building blocks encoding mAbs can be easily combined to produce Abs which bind specifically to multiple targets within the same protein. Overall, the synthetic Ab field is poised to revolutionize the mAb market and provide novel tools and treatments for human disease.

**Presentation:** Oral



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## The 6 Principles of Animal Research Ethics – Questions and application

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The recent publication of *Principles of Animal Research Ethics*, 2020, Oxford University Press by Tom Beauchamp and David DeGrazia outlines the Beauchamp-DeGrazia Framework of Principles (Beauchamp and DeGrazia, 2020). This presentation will explore the existing ethical gap during IACUC (or equivalent) review, even with the application of Harm/Benefit analysis and the 3Rs of Replacement, Reduction and Refinement. This gap exists because HBA and 3Rs do not include a robust enough framework for ethical debate on animal studies. Beauchamp and DeGrazia's analysis of the gap resulted in 3 premises: (1) sentient animals have moral status and therefore are not merely tools of research, (2) the only possible justification for (non-therapeutically) harming animals with moral status, including animal research subjects, is the prospect of substantial and otherwise unattainable social benefits, and (3) any permissible harming of animals in research is limited by considerations of animal welfare. In turn these premise lead to two core values of social benefit and animal welfare. The principle of Social Benefit in turn has 3 principles: (1) The Principle of No Alternative Method, (2) The Principle of Expected Net Benefit and (3) The Principle of Sufficient Value to Justify Harm. The Principles of Animal Welfare include an additional three principles: (1) The Principle of No Unnecessary Harm, (2) The Principle of Basic Needs and (3) The Principle of Upper Limits to Harm. This session will examine these new principles and what they can mean in the future, from the perspective of laboratory animal medicine, governance, ethics and philosophy.

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**Presentation:** Oral

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## Caring for those caring for research animals: Developing a global corporate resiliency building program

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In recent years, there is increased recognition of the impact that compassion stress and compassion fatigue have on mental health and well-being as occupational hazards for veterinary and research animal facility personnel. These are normal consequence of caring people giving to others while not taking time to care for themselves. Those working with research animals can be profoundly affected by their work, including feelings of grief and sadness, or a sense of loss that they may not be able to articulate. Left unaddressed, caregivers may become desensitized and numb, which can affect the animals that they are entrusted to care for, making this not only a human issue, but potentially an animal welfare issue. To address the effects of compassion stress and compassion fatigue, we developed and administered surveys, both internally and within the larger laboratory animal community in North America and Europe, looking at root causes and coping mechanisms to help individuals deal with feelings of compassion fatigue. These surveys were used to develop an impactful corporate program to meet the needs of personnel across multiple sites. The program provides training sensitizing management to compassion fatigue, their role in helping personnel deal with difficult situations at work, and ways to support building resiliency within their employees. A network of "resiliency building ambassadors" to extend the program to multiple sites across the organization, focusing on tools for building resiliency, peer counseling through empathetic listening, personal wellness, providing tributes to research animals worked with, enhancing communications during research projects, promoting animal adoption and rehoming programs. Focused training for frontline leaders, human resource representatives, and veterinary personnel to further support personnel and create an emotionally engaged culture within the facility. The program includes flexibility for sites to select content to ensure that culturally appropriate tools and materials are available across sites.

**Presentation:** Oral



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## Needs for application of air-liquid-interface models in risk assessment of inhaled compounds

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Technical developments have resulted in systems that can be used to expose cells via the air to mimic inhalation exposure. In parallel, *in vitro* models for the respiratory tract have been improved greatly to have more commonalities with the human respiratory tract. There are, however, some steps that need to be taken for the application of these *in vitro* models in human risk assessment. The main topics to address are how the dosing and the effect can be translated from human exposure to *in vitro* models and *vice versa*. Translation of human dose to *in vitro* concentration needs to consider airway deposition and deposition on the cells in the *in vitro* exposure system that is used for exposure. In addition, for application in risk assessment, also the *in vitro* concentration needs to be extrapolated to human inhaled concentration. The health effect, and thereby the *in vitro* model is driven by the toxicological hypothesis as the cell model should allow for measurement of the parameter of interest. For most *in vitro* effects the translation to human health effects remains a challenge. Probably, multiple readouts need to be combined to translate *in vitro* effects to human health effects. This presentation will introduce the session on the translation of inhaled concentrations and effects in Air-Liquid-Interface (ALI) cultured cell models to human effects. Information needs on deposition in human airways and in *in vitro* models will be addressed and the extrapolation of *in vitro* effects to human health effects will be discussed using an Adverse Outcome Pathway (AOP) approach.

**Presentation:** Oral

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## Microphysiological system coculture approach for bronchial lung and liver models for substance exposure studies

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The restricted complexity of current organ models and the lack of interorgan communication limits the predictability of cell cul-

ture-based assays for assessing the effects of substances in humans. Biology-inspired microphysiological systems provide pre-clinical insights into the absorption, distribution, metabolism, and toxicity of substances *in vitro* by using cocultures of human three-dimensional organotypic cultures.

Here, we describe a coculture of a human lung equivalent from the commercially available bronchial MucilAir™ culture and human liver spheroids from the HepaRG™ cell line. The coculture has been designed to address the potential toxicity of inhaled compounds and directly predict their effects and metabolism in a trans-organ environment. For this, we have developed a new HUMIMIC Chip design and created appropriate coculture conditions with regard to medium supply and oxygenation of the organ cultures. The tissue constructs were integrated into separate culture compartments of the closed circulatory perfusion system, interconnected by microfluidic channels, and cultivated for 14 days. This helped demonstrate that the viability and homeostasis of the tissue cultures could be maintained over longer cultivation periods. The morphology of MucilAir™ tissues was additionally evaluated by histological analysis and their functionality by measurement of transepithelial electrical resistance and cilia beating frequency. Furthermore, immunohistochemical analysis and quantification of albumin secretion helped verify liver tissue function in the coculture. The connection between lung and liver tissues and the ability of the liver to metabolize compounds present in the medium was demonstrated by using aflatoxin B1, a known hepatotoxicant and carcinogen. Indeed, aflatoxin B1 toxicity in chips containing lung and liver tissues was lower than that in chips containing lung tissues only.

In summary, the new HUMIMIC Chip setup enables exposure studies for investigating the toxic effects of inhaled substances on a trans-organ level, emulating more closely the systemic effects of the substance in the human body.

**Presentation:** Oral

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## European lessons learned from REACH submissions using NAM skin sensitisation data

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The REACH standard information requirements were amended in May 2017 to include sequential testing strategy when new information needs to be generated for a chemical substance. As a first step *in vitro/in chemico* studies need to be generated. In case the *in vitro/in chemico* methods are not applicable for the substance or the results obtained from such studies are not adequate for classification and risk assessment an *in vivo* study can be performed (Local Lymph Node Assay as a first choice of assay). In addition, the REACH regulation was amended to include infor-



mation on skin sensitisation potency for skin sensitising substances.

After the last registration deadline of 31 May 2018 for the lower tonnage substances (manufactured or imported at 1 to 100 tonnes per annum per registrant) a data mining exercise was performed to investigate the strategies that the registrants have taken for the endpoint of skin sensitisation. The results presented in this report are based on registration data extracted from the IUCLID database on 31 July 2019. This dataset includes practically all registrations submitted as part of the third REACH registration deadline in May 2018. From this data mining more in-depth assessment was performed on cases where *in vitro/in chemico* data was used to fill the REACH information requirement for skin sensitisation. The presentation will provide more in-depth information on the approaches used and conclusions made by the registrants, i.e., number of substances where solely *in vitro/in chemico* methods were used, number of substances where *in vitro/in chemico* methods were used in combination with *in vivo* methods, substances having *in vivo* results only, and what was the reasoning for the selected approaches.

**Presentation:** Oral

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## Culture of care at Novartis – The path for better science

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Through a multitude of peer-reviewed research, it has become clear that animal care and welfare is a critical factor in the quality of the scientific results obtained from animal research. Animal care and welfare staff have key insights to contribute to decision-making regarding housing, handling and euthanasia that can improve the quality of scientific results. A good culture of care engages staff and facilitates this collaboration. Developing and growing a culture of care requires openness, transparency and the commitment of the institution's senior leaders.

In order to ensure high animal welfare standards and a culture of care, Novartis established a global animal welfare organization in 2005. This organization sets, implements and monitors global animal welfare studies and procedures pursued internally and with external partners at a local and global level. The team works in close collaboration with our global laboratory animal service organization that provides daily care to our animals, including 24/7 access to specialty trained veterinarians.

In addition, Novartis is committed to the 3Rs principles of Reduction of animal numbers, Refinement (improving the animal's experience in research) and Replacement (replacement of animals with non-animal methods).

Examples for a good culture of care will be given for

1. Global and local annual 3Rs awards

2. Implementation of better handling techniques
3. Compassion fatigue
4. Boxless events
5. Celebration of our commitment to ethical engagement of animals in research through the annual Global Biomedical Research Awareness Day (BRAD)

**Presentation:** Oral

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## An international data crowdsourcing project to understand and minimise aggression in group-housed male laboratory mice

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Aggression in group-housed laboratory mice is a serious animal welfare concern. Further understanding of the causes of aggression could have a significant positive impact on a large number of animals worldwide. The NC3Rs led a crowdsourcing project for animal technicians to collect data on the prevalence and potential triggers for mouse aggression. The crowdsourcing approach allowed data to be collected from multiple institutions and is the first time such an approach has been applied to a laboratory animal welfare problem. Technicians observed group-housed, male mice during daily routine cage checks and recorded all incidents of aggression-related injuries. Data was anonymised and analysed to calculate prevalence and identify factors contributing towards cage aggression. In total, 44 facilities from nine countries participated in the study and data was collected by 143 animal technicians. A total of 788 incidents of aggression-related injuries were reported across a sample population of 137,580 mice. The mean facility-level prevalence of aggression-related incidents reported across facilities was equivalent to 15 in 1,000 mice. Key factors influencing the prevalence of aggression included strain; number of mice per cage; how mice were selected into a cage; cage cleaning protocol; and transfer of nesting material. The study results, along with the current published literature, were used to formulate practical recommendations for minimising aggressive behaviour in mouse facilities (Lidster et al., 2019).

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**Presentation:** Oral



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## Innovative strategies in biomedical research: Which models?

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Animal models have been traditionally used in biomedical research to recapitulate human disease features and develop new drugs, as they are generally supposed to resemble some of the major hallmarks of human diseases. However, these animals do not develop the disease as it occurs in humans, and their use has not paved the way to the development of drugs effective in human patients for many highly prevalent non-communicable diseases, such as Alzheimer disease. Indeed, despite conspicuous research and economical endeavors, the clinical failure rate in drug development still remains very high, with an overall likelihood of approval from Phase I of about 9.6%. On the other hand, enhanced human clinical trials utilizing micro-dosing, and more representative study populations and durations, as well as surrogate human tissues, advanced imaging modalities and human epidemiological, sociological and psychological studies, may increase our understanding of illness etiology and pathogenesis, and facilitate the development of safe and effective pharmacologic interventions. Particularly when human tissues are used, non-animal models may generate faster, cheaper results, more reliably predictive for humans, whilst yielding greater insights into human biochemical processes. A first effort to gather existing knowledge about non-animal models of highly prevalent human diseases has been made by the Joint Research Centre of the European Commission. The final goal is to disseminate and improve knowledge sharing on potentials and limitations of human based models at different levels: scientific communities, universities and secondary schools, national committees for animal welfare and the public at large.

**Presentation:** Oral

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## Tradition, not science, is the basis of animal model selection in drug development

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National and international laws and regulations exist to protect animals used for scientific purposes, e.g., Directive 2010/63/EU. Therefore, we investigated how animal model selection was reported in project application forms for animal procedures for scientific purposes (Veening-Griffioen, 2021). We evaluated the choice of a specific animal model using thematic content analysis in project application forms, issued in 2017-2019 by the national Central Authority in the Netherlands. In total, 125 animal models for translational and applied research, from 110 project applications were assessed. Explanations to select a specific model included: the model's availability (79%); the availability of expertise (62%); and the model showing similar disease pathology/symptoms (59%). Therefore, current selection of a specific animal model seems to be based on tradition rather than its potential predictive value for clinical outcome. The applicants' explanations for the implementation of the 3Rs principles (replacement, reduction and refinement) as to the animal model were often un-specific: replacement was achieved by using data from prior *in vitro* studies, reduction by optimal experimental design and any statistics, and refinement by reducing of discomfort. Additionally, due to the need for a test model with high complexity (47%) and intactness (30%), the full replacement of animal models with alternative (non-live animal) approaches was thought unachievable. Without a clear, systematic and transparent justification for the choice of a specific animal model, the likelihood of poorly translatable research remains. It is not only up to the researcher to demonstrate this, as ethical committees and funding bodies can provide positive stimuli to drive this change.

### Reference

Veening-Griffioen, D. H., Ferreira, G. S. et al. (2021). *ALTEX* 38, 49-62. doi:10.14573/altex.2003301

**Presentation:** Oral



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## Cellular cross-talk-induced secretion of interleukin-10 (IL-10) in an organotypic human melanoma model directs monocyte differentiation to an M2-like phenotype

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The melanoma microenvironment promotes immune escape and tumor progression, contributing to failure of melanoma therapies in a large group of treated patients. While two-dimensional (2D) cultures lack tissue context, animal models poorly predict human immune responses, with the result that potential new drugs fail in the clinical setting. This highlights a pressing need for more physiological human melanoma models. Here, we established an *in vitro* three-dimensional (3D) reconstructed organotypic human melanoma (Mel-RhS) model to investigate tumor formation and progression over a six-week period. Tumor nests were observed to develop over time and to spread towards the dermis, disrupting the basement membrane. This coincided with the secretion of matrix metalloprotease 9 (MMP-9) by melanoma cells. These features resemble the early stages of *in vivo* melanoma invasion. Interestingly, while interleukin-10 (IL-10) could not be detected in monolayers of the employed SK-MEL-28 melanoma cell line, the cellular crosstalk in the 3D model led to IL-10 expression by the melanoma cells, as well as by the surrounding keratinocytes and fibroblasts. This resulted into a higher secretion of IL-10 in comparison to healthy controls, thus suggesting the generation of an immune suppressive Mel-RhS microenvironment. Indeed, Mel-RhS-derived culture supernatants interfered with monocyte-to-dendritic-cell differentiation, skewing them towards a tolerogenic M2-like macrophage phenotype instead, which was partly prevented upon IL-10 blockade. Thus, the Mel-RhS 3D configuration recapitulated a role for IL-10 in metastatic immune escape through misdirected myeloid differentiation, demonstrating the potential of the Mel-RhS in research on melanoma development and invasion in the setting of an immune competent model. In the future, the Mel-RhS may also provide a novel *in vitro* tool for preclinical testing of immune modulatory therapeutics.

**Presentation:** Oral

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## Do we act like car salesmen or airline pilots? Preparing for robust and humane research

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Many explanations for the so-called “reproducibility crisis” in science focus on the “mathematical” aspects of planning and conducting preclinical experiments, such as the appropriateness of statistical analyses, or the failure to prevent bias. Whilst undeniably important, this approach ignores the many animal-related factors which produce artefacts and reduce both internal and external validity. Unfortunately, it is more difficult to produce metrics which reflect these factors or attempts to avoid them.

The last few decades have seen major advances in most aspects of laboratory animal husbandry and care. It is unreasonable to expect scientists (who use animals in their research – but whose main focus is naturally elsewhere) to be fully aware of current “best” practice. Scientists need guidance before embarking on animal-based experiments and this should be provided at the earliest possible stage by key personnel from the animal facility in which the research is to be conducted.

The PREPARE guidelines for planning animal research and testing are an attempt to provide an overview of the issues which should be considered, from day one. Unlike reporting guidelines, they are not intended to be a mandatory requirement. They were conceived over a 30-year period to be a voluntary aide memoire for scientists. The PREPARE checklist, currently available in 25 languages, provides a quick overview of 15 central topics, but importantly, the PREPARE website expands on each of these topics and provides links to the latest advances within each area (<https://norecopa.no/PREPARE>). A 3-minute cartoon film describing the guidelines and drawing parallels from the aviation industry, is also available, with optional subtitles in 8 languages (<https://norecopa.no/PREPARE/film>).

**Presentation:** Oral



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## Rethinking validation: Building confidence in human models through biologically based design

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Validation of new approach methods is currently based on comparisons to *in vivo* animal data, assessing how well these alternative methods reproduce past results of organism level toxicity in intact animals. However, the cost and time associated with these traditional validation approaches are prohibitive to rapid adoption of new approaches, and reliable *in vivo* models are not available for all toxicological endpoints of interest. Thus, we must consider new strategies for validation of new approach methods. One such approach is a biologically based validation process taking advantage of the advances in our understanding of biology and medicine to identify the key components of biological pathways (Adverse Outcome Pathways; AOPs) and incorporation of these key events into the test system. Thus, the focus is on the design of the system and strategic testing of the system with meaningful controls. Case studies will be presented to demonstrate the value in using AOP-structured approaches to increase the utility and predictivity of *in vitro* assays, not only for potential hazard identification, but also for quantitative risk assessment, e.g., setting a point of departure (PoD).

**Presentation:** Oral

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## Perceived barriers to implementing refinements in euthanasia for rodents

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Carbon dioxide (CO<sub>2</sub>) inhalation induces negative affective states (including anxiety, fear and distress) in laboratory rodents, but many laboratories continue to use this as an euthanasia method. Refinements that are less aversive than CO<sub>2</sub>, such as overdose via inhalant anesthetics, are not widely adopted. To better understand perceived barriers to adopting alternative methods, we conducted an online survey of Canadian (n = 209) and European (n = 410) laboratory animal professionals and researchers. Very few participants (14% in Canada and 22% in Europe) had favorable views on the use of CO<sub>2</sub>, but approximately 50% of responders still used this gas to euthanize laboratory rodents. Participants' responses focused on the animal's experience, practical considerations, and how these issues should be balanced. Many

participants (~50%) believed that alternatives were available that could be considered an improvement over CO<sub>2</sub>, but they perceived barriers to implementing these alternatives. These barriers included: 1) financial, 2) regulatory, 3) safety, 4) research, and 5) institutional culture constraints. Few participants described the lack of scientific evidence for refinements as an issue. We conclude that participants are concerned about the effects on CO<sub>2</sub> on animal welfare, but that perceived barriers to implementing alternatives appear largely related to operational limitations. These results suggest that new research should focus on how these institutional barriers may be overcome. We speculate that addressing these barriers may improve the adoption of other refinements to laboratory environments.

**Presentation:** Oral

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## Bioengineered 3-dimensional lung organoids as an alternative to patient-derived xenograft models of small cell lung cancer

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Lung cancer is the major cause of cancer death in both men and women worldwide showing the lowest 5-year survival rate among all cancer types. Small cell lung cancer (SCLC) is a highly metastatic, neuroendocrine sub-type representing about 10% of all lung cancers. Therapeutic discovery for SCLC is extremely challenging due to the relapse of the disease with chemoresistance. Most of the drugs fail in the pre-clinical and clinical trial phase because the existing drug testing models are unable to recapitulate the actual tumor pathophysiology. Patient-derived xenografts (PDX) are considered the gold standard for drug development in pre-clinical settings, but this has several limitations, including chances of engraftment failure, long development timeline, dissimilarity of tumor microenvironment between human and murine models, substantial cost, and a huge sacrifice of animal lives.

To address these issues, here, we present a bioengineered 3-dimensional (3D) lung SCLC organoid model to study tumor growth kinetics and response to chemotherapy. We custom made functionalized alginate microbeads coated with human lung fibroblasts and heterogeneous SCLC cell lines in a specially de-



signed bioreactor to build a 3D model of distal human lung with SCLC tumors. We found that SCLCs in the 3D model proliferated and invaded the microbeads and formed co-culture 3D tumors within a very short duration (72 h). We compared this bio-engineered model with patient tumors and found it to reproducibly recapitulate the pathology and immunophenotyping of the patient tumors. When treated with dose courses of chemotherapy drugs, Cisplatin and Etoposide, alone or in combination, the model showed significantly higher drug resistance than the 2D cell cultures with a relapsing pattern similar to that of the existing PDXs. Being amenable to high throughput drug screening, this co-culture model can be a faster and advanced alternative to animal PDX models to study SCLC.

**Presentation:** Oral

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## Applying the 3Rs principles in wildlife research through non-invasive methods

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Research in ecology and wildlife biology remains crucial for increasing our knowledge and improving species management and conservation in the midst of the current biodiversity crisis. However, obtaining information on population status often involves invasive sampling of a certain number of individual animals. Marking and sampling practices include taking blood and tissue samples, toe-clipping of amphibians and rodents, or using implants and radio-transmitters – techniques that can negatively affect the animal. Wildlife research may then result in a fundamental conflict between individual animal welfare and the welfare of the population or ecosystem, which could be significantly reduced if non-invasive research practices were more broadly implemented. Implementation of non-invasive methods could be guided by the so-called 3Rs principles for animal research (Replace, Reduce, Refine), which were proposed by Russell and Burch more than 60 years ago (Russell et al., 1959) and have become a part of many animal protection legislations worldwide. However, the process of incorporating the 3Rs principles into wildlife research has been unfortunately rather slow and their importance overlooked (Zemanova, 2017, 2019). I will provide an overview of the most common practices in wildlife research, discuss their potential impact on animal welfare, and present available non-invasive alternatives (Zemanova, 2020).

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**Presentation:** Oral

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## A simple-to-use model to work purposeful and focused with culture of care

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Culture of Care can be a powerful and efficient tool to ensure, promote and advance animal welfare and the practical application of the 3 Rs – or it can be a meaningless phrase with no impact on these parameters. Approaches on how to work with Culture of Care has recently been published by Robinson et al. (2019) and Hawkins and Bertelsen (2019). Each user-establishment has its own and unique Culture of Care which requires a tailor-made attention to address the relevant challenges and issues.

This presentation takes the differences of each user-establishment into account and provides an universally applicable and operational overview of a working model of 1) how to assess the actual Culture of Care of the individual user-establishment and how to identify relevant topics and challenges required for change, 2) how to work determined and focused with Culture of Care by deploying different approaches at different levels to manage and direct the required initiatives for changes and 3) how to measure the effectiveness of the Culture of Care – and the effect of subsequent implemented changes – in terms of delivering a positive impact on animal welfare and the practical application of the 3 Rs, e.g., by using relevant Key Performance Indicators (KPIs). The model is simple and coherent and yet comprehensive as it covers the most relevant aspects of working with Culture of Care.

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**Presentation:** Oral



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## All-human, dynamic, *in vitro*, 3D blood brain barrier models for drug delivery and cancer metastasis studies; basal laminae protein types define TEER and transient opening can be demonstrated, reflecting human xenograft model findings

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The blood brain barrier (BBB) functions as a barrier to toxins reaching the brain. It also, however, hampers delivery of therapeutics for neurological diseases – including malignant tumours – to the brain. Attempts to model the human BBB *in vitro* in order to closely investigate the functional complexity of the barrier have largely been based around the use of vascular endothelial cells derived not from the brain, but from other organs, or, indeed even from other species (both *in vitro* and *in vivo*). We have engineered 3D dynamic (shear stress/flow) models from human brain-derived cells (endothelial cells, astrocytes and pericytes) and vascular basal lamina proteins which yield hitherto unreported high TEER levels of > 1,200 ohms, akin to those reported *in situ*. (Maherally et al., 2018). These values were achieved by the specific use of perlecan and agrin (where previously knock down of these proteins in laboratory mice had been reported to totally disrupt the BBB). Our new models, constructed under human serum supplementation and oxygen levels of 0.1% to 20% have been successfully used to investigate NSCLC metastasis to the brain (Jassam et al., 2015, 2017, 2019) as well as in *in vitro* experiments to “verify” the transient opening of the barrier in human melanoma to brain metastasis in xenografts by way of A16ApoE (Aasen et al., 2019). These 3D all-human *in vitro* BBB models are not only more representative of the human brain *in situ* than *in vitro* and *in vivo* animal models, but they pave the way for accurate pre-clinical assessment of therapeutic drug delivery to the brain for a wide range of neurological disorders and diseases, including malignant brain tumours.

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**Presentation:** Oral

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## Multi-endocrine disruptors screening in zebrafish

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*In vitro* tools are inexpensive and scalable for high-throughput platforms; however, they pose low relevance to vertebrate species. On the other hand, work with adult animals is expensive and represents ethical issues. Small fish like zebrafish (*Danio rerio*) are an excellent alternative to *in vitro* and *in vivo* model. They offer a unique experimental system where screening assays can be performed at the whole animal level, being at the same time compatible with the 3R principles (replacement, reduction and refinement in animal testing).

Endocrine disruptors (EDCs) are chemicals that by interfering with the endocrine system can have an adverse effect at developmental, neurological, immune and reproductive levels. Thyroid Disrupting (TD) compounds specifically alter the function of the thyroid gland through the interference with the synthesis, transport and/or binding of the thyroid hormones. The negative impact of EDCs is becoming a real public health issue, therefore the necessity of tests to assess the potential risk of new chemicals before they are marketed is increasing.

The zebrafish is currently used as a model for the evaluation of acute and developmental toxicity and for the screening and testing of potential endocrine disruptors (EDCs), as described in the OECD Guidelines. The two major endpoints used to evaluate EDCs, vitellogenin concentration and change in sex ratio, have several limitations. With the purpose of expanding the number of tests available to identify estrogenic, androgenic, and thyroid disrupting substance, we evaluate gene expression of 4 biomarkers in 5 dpf zebrafish larvae after exposure, from 48 hpf to 10 compounds reported as EDCs. A transgenic strain, expressing fluo-



rescence coupled to the induction of thyroglobulin was also used to evaluate 9 environmentally relevant TD substances.

This screening methodology showed to be a sensitive and cost-effective assay to screen and evaluate potential EDCs chemicals.

**Presentation:** Oral

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## An analysis of the limitations and uncertainties of *in vivo* developmental neurotoxicity testing and assessment to identify the potential for alternative approaches

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Limitations of regulatory *in vivo* developmental neurotoxicity (DNT) testing and assessment are well known, such as the 3Rs conflict, low throughput, high costs, high specific expertise needed and the lack of deeper mechanistic information. Moreover, the standard *in vivo* DNT data variability and the experimental animal to human real-life extrapolation is uncertain. Here, knowledge about these limitations and uncertainties is systematically summarized and visualized. We also outline a hypothesis how alternative, fit-for-purpose Integrated Approaches to Testing and Assessment (IATAs) for DNT could improve current standard animal testing: Relative gains in 3Rs compliance, reduced costs, higher throughput, improved basic study design, higher standardization of testing and assessment and validation without 3Rs conflict, increasing the availability and reliability of DNT data. This could allow a more reliable comparative toxicity assessment over a larger proportion of chemicals within our global environment. The use of early, mechanistic, sensitive indicators for potential DNT could better support human safety assessment and mixture extrapolation. Using kinetic modelling ideally these could provide – eventually context dependent – at least the same level of human health protection. Such new approaches could also lead to a new mechanistic understanding for chemical safety, permitting determination of a dose that is likely not to trigger defined toxicity traits or pathways, rather than a dose not causing the current apical organism endpoints. The manuscript shall motivate and guide the development of new alternative methods for IATAs with diverse applications and support decision-making for their regulatory acceptance.

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**Presentation:** Oral

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## The international regulatory roadmap to enhance developmental neurotoxicity testing

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Since its release, the use of the OECD *in vivo* developmental neurotoxicity (DNT) test guideline has been limited in most regulatory sectors, making difficult the access to DNT data that is required to guide the safety assessment of chemicals. This fact increased the concern related to the DNT endpoint within the regulatory community as well as the society that witnessed an increase of the neurodevelopmental disorders the last decade that could not only be explained by genetic factors. Regulators and scientists identified as priority the need to better capture DNT mechanistic information for chemicals through the development of rapid and high throughput tests that could eventually lead to an improved understanding of chemically induced DNT and support the interpretation of available *in vivo* results.

This presentation aims to highlight how a series of workshops/meetings on DNT in the last decade led to a consensus among stakeholders that there is need for an *in vitro* DNT testing battery that relies on key neurodevelopmental processes (e.g., proliferation, differentiation, synaptogenesis, etc.) and is complemented by alternative species (e.g., zebrafish) assays. The presentation also explores the implementation of the battery within mechanistically informed integrated approaches for testing and assessment (IATA) through relevant case studies.

Overall, the presentation will focus on the OECD DNT guidance on *in vitro* testing battery that is under developmental that is an international effort and the activities that are ongoing to facilitate its finalization.

**Presentation:** Oral



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## Limitations and uncertainties of acute fish toxicity assessments can be reduced using alternative methods

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Information about acute fish toxicity is routinely required in many jurisdictions for environmental risk assessment of chemicals. This information is typically obtained using a 96-hour juvenile fish test for lethality according to OECD test guideline (TG) 203 or equivalent regional guidelines. However, TG 203 has never been validated using criteria currently required for new test methods including alternative methods. Characterization of the practicality and validity of TG 203 is important to provide a benchmark for alternative methods. This contribution systematically summarizes the available knowledge about limitations and uncertainties of TG 203, based on methodological, statistical, and biological considerations. Uncertainties stem from the historic flexibility (e.g., use of a broad range of species) and constraints of the basic test design (e.g., no replication). Other sources of uncertainty arise from environmental safety extrapolation based on TG 203 data. Environmental extrapolation models, combined with data from alternative methods, including mechanistic indicators of toxicity, may provide at least the same level of environmental protection. Yet, most importantly the 3R advantages of alternative methods allow a better standardization and characterization, and an improved basic study design. This can enhance data reliability and thus facilitate the comparison of chemical toxicity, as well as the environmental classifications and prediction of no-effect concentrations of chemicals. Combined with the 3R gains and the potential for higher throughput,

a reliable assessment of more chemicals can be achieved, leading to improved environmental protection.

### Reference

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**Presentation:** Oral

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## An *in vitro* 3D fracture gap model as a tool for preclinical testing procedures

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Approximately 10% of fractures lead to significant fracture healing disorders. To address the clinical need and to study the influence and efficacy of potential therapeutics, we developed an *in vitro* 3D fracture gap model (FG model), consisting of human cells and composed of an *in vitro* fracture hematoma model (FH consisting of peripheral blood and MSCs) co-cultivated with scaffold-free bone-like constructs (SFBC), correspondingly produced from autologous MSCs.

To proof the validity of the established *in vitro* FG model, we here studied the impact of (i) the bone-like construct on the *in vitro* FH model with regard to its osteogenic induction capacity and (ii) the iron chelating hypoxia-inducible factor stabilizer deferoxamine (DFO) known to enhance bone healing.

We co-cultivated the FH and the SFBC for 48 h under hypoxic conditions (n = 3). To analyze the impact of the SFBC on the *in vitro* FH model, we cultivated the FG models in medium w/o osteogenic supplements. Furthermore, we incubated the FG models w/o 250 µmol DFO. After incubation, we evaluated gene expression of osteogenic (RUNX2, SPP1), angiogenic (VEGF, IL8), inflammatory (IL6, IL8) and hypoxia-adaptation (LDHA, PGK1) markers and secretion of cytokines/chemokines.

Here, we demonstrate that the SFBCs induced the upregulation of osteogenic markers within the FHs irrespective of the supplementation of osteogenic factors in the medium. Furthermore, we observed an upregulation of hypoxia-related, angiogenic and osteogenic markers under the influence of DFO and the downregulation of inflammatory markers compared to the untreated control, also confirmed on protein level.

In summary, we demonstrate that our *in vitro* FG model provides all osteogenic cues to induce the initial bone healing pro-



cess, supplementary enhanced by DFO. Therefore, we believe that our model is able to mimic the human fracture gap situation in order to study the influence and efficacy of potential therapeutics.

**Presentation:** Oral

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## Developing an integrated approach to testing and assessment for non-genotoxic carcinogens

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There is no regulatory requirement to address non-genotoxic endpoints, thus in jurisdictions where the rodent cancer bioassay is not used or waived, it appears likely that non-genotoxic carcinogens (NGTxC) remain unidentified. A project for the development of an Integrated Approach to Testing and Assessment (IATA) for NGTxC started at the OECD in 2015. IATA are science-based approaches for chemical hazard characterization that rely on an integrated analysis of existing information coupled with the generation of new information using testing strategies. The project under development is led by the United Kingdom and supported by an OECD group of experts in the field. It aims to identify and prioritize promising assays for inclusion in an IATA and develop an IATA that will assist regulators in their assessment of NGTxC in order to address the regulatory gap. The presentation will describe the project and methodology used and will provide an update on the status of the work and expected next steps.

**Presentation:** Oral

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## Innovative technologies integrating standardized regulatory test methods: Challenges and perspectives

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Modern technologies supported by good science are progressively bringing innovative solutions to move away from animal testing. On the one hand, innovation comes with costs and the resulting intellectual property needs both to be protected and to remain accessible to users. OECD promotes good licensing prac-

tices for protected elements in the area of chemical safety testing (OECD, 2019a), and accompanies the transition to innovative methods in many different ways. This support is important for innovative thinkers to continue to engage and propose science-based solutions to regulators.

On the other hand, the regulatory community requires reliable, robust and transparent methodologies that they can rely upon for the safety evaluation of chemicals. These two communities need to dialogue early on to develop solutions that meet all standards and receive acceptance in practice.

Recently, cloud-based prediction models have introduced the premise of machine-learning algorithms and artificial intelligence into future regulatory standard test methods. The OECD Test Guidelines and Good Laboratory Practice frameworks are adapting and contemplating additional principles, guidance and guidelines needed by countries, to ensure that Mutual Acceptance of Data continues to be a reality that countries and industry can benefit from (OECD, 2019b).

### References

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- OECD (2019b). Saving Costs in Chemicals Management: How the OECD Ensures Benefits to Society. OECD Publishing, Paris. doi:10.1787/9789264311718-en

**Presentation:** Oral

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## More than 3Rs – The 3Vs and the ethics of animal research

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The use of animals for research is regulated on the explicit understanding that no unnecessary harm is imposed on animals. This is the legal basis of the 3Rs principle (replace, reduce, refine), which serves to minimize harm to animals in research. Researchers using animals must be able to explain why they cannot achieve the expected knowledge without using animals (replace), by using fewer animals (reduce), or by using less harmful procedures (refine). Whether the use of animals is legitimate, however, depends also on the expected benefit of the research. Unless study findings are scientifically valid and reproducible, animals may be harmed for inconclusive research. Indeed, research on the quality of research (meta-research) has revealed considerable threats to the validity and reproducibility of animal research. These include poor animal models threatening the validity of what is being studied (construct validity), a lack of scientific rigor



in experimental design and conduct questioning the inferences drawn from the results (internal validity), and rigorous genetic and environmental standardization limiting the generalizability and reproducibility of the results (external validity). I therefore propose a more explicit assessment of these three key aspects of the scientific validity of animal research (construct validity, internal validity, external validity) when reviewing grant proposals, study protocols, and publication manuscripts. This is what I refer to as the 3Vs principle (Würbel, 2017). As with the application of the 3Rs principle, there is no need for a fixed checklist approach. Instead, appropriate criteria for assessing the 3Vs could be defined according to the decisions to be taken (e.g., project funding, protocol approval, publication). Together with the 3Rs, promoting the 3Vs in animal research will help us avoid wasting animals for inconclusive research and imposing unnecessary harm on research animals.

#### Reference

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**Presentation:** Oral

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## Overview of research program for user-driven MPS development in Japan

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In 2017, the National MPS project supported by the Japan Agency for Medical Research and Development (AMED), called AMED-MPS was launched in Japan.

AMED-MPS consists of 3 research programs: 1) Cell supply and MPS model development of four organs: liver, gut, kidney and blood-brain barrier, 2) Device Manufacturing, and 3) Standardization for quality control and regulatory development, Central Research Center (CRC), and headquarters (HQ): decision-making body in AMED-MPS.

Noteworthy, senior managers and researchers in pharmacokinetics, safety/toxicity fields from domestic pharmaceutical companies participate in the project as a member of HQ and research partners.

Each research program, CRC and end-users are closely collaborating with all program members and are conducting research and development to transfer newly-developed MPS technologies from academia into practical use in end-users.

Here, we present the overview of AMED-MPS and a case example of user-driven MPS research and development.

#### Reference

Marx, U., Akabane, T., Andersson, T. B. et al. (2020). *ALTEX* 37, 365-394. doi:10.14573/altex.2001241

**Presentation:** Oral

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## Tools to support application of physiologically based kinetic modelling in safety assessment

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The development of non-animal alternatives in safety assessment is a priority area across multiple sectors. Read-across is increasingly being used, whereby information from data-rich (source) chemicals can be leveraged to infer information for data-poor (target) chemicals, provided that source and target chemicals are suitably "similar". In order for any chemical to elicit an effect, the chemical must possess intrinsic activity and be able to reach the relevant site of action at an appropriate concentration. Physiologically based kinetic (PBK) models can be used to predict time-concentration profiles for chemicals in blood and/or organs so enabling a more accurate description of internal exposure that can inform safety-assessment decisions. PBK models require a wide range of data and are time-consuming to generate. As an increasing number of models have now been published in the literature, these can be used as templates for other chemicals of interest – as demonstrated by Lu et al. (2016) and Pains et al. (2021). Development of this concept requires information regarding the chemicals for which such models exist, and a method to identify which of these chemicals is "similar" to the target; this EPAA-funded project, is addressing these issues. A systematic review of published PBK models, has been completed, resulting in a readily searchable dataset of such models identified for over 1,400 unique chemicals. The chemical space coverage of the models, compared to the chemical space of food additives, drugs and industrial chemicals has been ascertained. Work to develop a method by which appropriate "similar" chemicals can be identified is on-going and preliminary results are discussed.

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*Disclaimer: The views expressed in this abstract are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.*

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**Presentation:** Oral

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## Identifying human thyroid disruptors in the 21<sup>st</sup> century needs a real paradigm shift in risk assessment approaches

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The enhanced attention for chemical-induced disruptive endocrine pathway modulation leading to adverse health effects has resulted in the incorporation of mandatory endocrine parameters into existing guideline-based animal studies for chemical safety assessment. The interpretation of endocrine parameter changes is challenging since they offer mechanistic insight but are not indicative of adversity *per se*. They should be causally related to an observed adverse outcome in order to infer that the endocrine change was indeed indicative of adversity. Hormone systems guard homeostasis in the body, providing continuous adaptation to the ever changing external and internal environment. Changes within homeostatic ranges are not adverse. Existing animal study protocols require only limited assessment of adversity, so that observed endocrine changes cannot always be readily interpreted as either adaptive or adverse. Likewise, a host of *in vitro* assays enable monitoring effects on isolated elements of the hypothalamic-pituitary-thyroid hormone axis, but do not inform about an integral adverse effect at the level of the intact individual. In a current CEFIC-LRI sponsored project a quantitative adverse outcome pathway (qAOP) is designed based on a study in pregnant rats in which the pathway connecting hepatic metabolism changes, consequent thyroid hormone axis modulation, and downstream adverse effects on perinatal brain devel-

opment, is mapped in great detail. This multi-dose-level study was designed to enable conclusions about quantitative limits between adaptive and adverse changes in the thyroid system, and which parameters should be assessed as essential to make that verdict. The qAOP will inform the design of an *in silico* prediction tool for liver-thyroid-brain development disruption and which *in vitro* assays are required to feed the *in silico* tool for toxicity predictions in an animal-free strategy. The application of *in vitro* assays and computational models in regulatory toxicology could revolutionize endocrine data interpretation and chemical hazard and risk assessment.

**Presentation:** Oral

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## (Q)SARs in the AOP-Wiki and OECD IATA case studies project: A brief snapshot

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Constant efforts to develop more efficient Quantitative Structure-Activity Relationship ((Q)SAR) techniques and models aim to effectively improve drug discovery and predictive (eco)toxicology applications, among many others. The use of QSARs is also well-accepted by the OECD to characterize Molecular Initiating and Key Events (MIE, KE) of Adverse Outcome Pathways (AOPs), or as part of Integrated Approaches to Testing and Assessment (IATA) (OECD, 2016). Considering the potential applications of (Q)SARs, it was decided to explore their effective use, as a predictive method, within the AOP-Wiki, and the published OECD IATA Case Studies, also including the 12 Defined Approaches (DAs) for assessing Skin Sensitization (OECD, 2017). At time of writing, among the 1285 MIEs/KEs totally available in the AOP-Wiki, 946 have an empty "How it is measured and detected" section. Of the remaining 339 MIEs/KEs, only 11 were found to report computational applications as detection methods, such as SAR, molecular docking, *in silico* homology modelling, and a computational model simulating a biological environment. Whereas a good implementation of (Q)SARs was noticed in the IATAs and DAs, since 11 of the 15 IATA and 7 of the 12 DA case studies embedded the use of *in silico* tools, such as the OECD (Q)SAR Toolbox, TIMES-SS, and others. Overall, this analysis shows that the use of (Q)SARs is still underutilized, especially for AOPs, despite the wealth of information and the



models available in the literature. Indeed, further investigation of the literature explores the availability of existing (Q)SARs for the prediction of selected MIEs and KEs lacking measurement methods.

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**Presentation:** Oral

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## Colinear Hox gene expression in the neural embryonic stem cell test (ESTn) defines its biological domain and reveals effects of compounds

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Hox genes are a family of highly conserved genes expressed in a colinear manner along the neural tube, defining the body plan and specificity of (neural) cells during development. Recent studies have shown that embryonic stem cells harbor a self-organizing capacity that show colinear expression of Hox genes *in vitro*. In this study we show that the neural embryonic stem cell test (ESTn) also presents this typical wave of Hox gene expression. This may provide a useful read-out in ESTn for the effects of chemicals on early neural differentiation. Using our existing microarray data set of the first seven days of ESTn differentiation we could define the developmental domain of the test based on gene expression of morphogens, signaling proteins as well as Hox genes. During embryonic body formation in the first three days of differentiation, key signaling molecules for anterior-posterior patterning such as Fgf8 and Wnt3 were upregulated. By adding exogenous retinoic acid for two days, a wave of Hox gene expression was unleashed, which presented a similar expression pattern as *in vivo*. After seven days of differentiation, Hox1-9 genes were

expressed, indicative for the brain to thoracic region of the spinal cord. Exposure of ESTn to chemicals that affect early neural differentiation showed a typical regulation of Hox genes. For example, valproic acid, cyproconazole, hexaconazole and flusilazole upregulated Hox4 and 5 genes and downregulated Hox8 and 9 genes, while carbamazepine showed an opposite trend. These results suggest that the latter compound accelerated differentiation, while the former compounds inhibited differentiation. This study provides insight in the biological domain of ESTn and the effects of chemicals on early neural differentiation. This method may be applied to other stem cell-based neural *in vitro* systems and may be useful in a testing strategy for screening chemicals for their neurodevelopmental toxic potential.

**Presentation:** Oral

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## A walk through 10 years of CAAT-Europe's highlights: (2012) A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing

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In 2012, a landmark paper appeared in ALTEX, authored by David Basketter and co-authored by 34 scientists from 8 European countries and the US. The report is introduced by a well-known bon mot of Winston Churchill "This report, by its very length, defends itself from the risk of being read", already indicating that it is a very comprehensive summary of the "alternative" systems, methods and technologies existing at the time of appearance of the report, aiming to ultimately replace animal experimentation. The following endpoints were addressed in the paper: toxicokinetics, skin sensitization, repeated dose toxicity, carcinogenicity, and reproductive toxicity. It is not the description of the state of the art, which makes this now highly cited report so valuable – not unexpectedly there were quite a number of new developments in the field; it is the description of information gaps and the general and partly detailed advice, where future research in the field should be directed at. This clearly assisted in the guidance of research in subsequent large European cooperative FP7 and Horizon2020 projects including ChemScreen, Seurat-1, and EU-ToxRisk. This presentation will not aim, however, to summarize the Basketter et al. paper, but rather give a personal view on where research in the field of "alternatives" might go.

## Reference

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for the development of alternative (non-animal) methods for systemic toxicity testing. *ALTEX* 29, 3-91. doi:10.14573/altext.2012.1.003

**Presentation:** Oral

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## A refinement Wiki

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Discussions about improving animal welfare and experimental design are an everyday part of the lives of all who use or care for animals in research. Many suggestions for improvements are easily accessible in textbooks or scientific papers. However, these suggestions are often more general in nature.

A number of discussion forums now exist, where specific refinements and new ideas can be shared. They allow discussions of techniques which do not warrant publishing as a scientific paper at that stage. However, many forums do not have easily searchable archives, so valuable ideas can easily be forgotten over time.

To bridge the gap between scientific papers and discussion groups, we have constructed a Refinement Wiki (<https://norecopa.no/Wiki>). We foresee a number of uses:

1. Rapid dissemination of refinement techniques where resources or interest in writing full-scale scientific papers are unavailable
2. As a hub where those investigating the effects of a potential refinement strategy in a multi-lab study can identify collaborators
3. Creation of pages encouraging colleagues to share experiences or develop new strategies to solve a problem the contents of the Wiki are not curated. The quality of the Wiki is determined by registered bona fide members of the research animal community. No one else can add, delete or comment upon material.

The Wiki is an integral part of Norecopa's website: <https://norecopa.no>. Wiki content is retrievable from Norecopa's main search engine. In addition, the Wiki has its own search engine. We have written a simple instruction manual to keep the threshold for adding new content as low as possible.

We hope that this Wiki will help to accelerate the introduction of refinement methods. It is now up to the community to judge

whether this initiative is worthwhile. Those interested in adding content to the Wiki may contact Adrian Smith (adrian.smith@norecopa.no).

**Presentation:** Oral

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## EPAA efforts to promote and build confidence in the use of 3Rs

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The EPAA (European Partnership for Alternative Approaches to Animal Testing) operates since 2005 as a voluntary collaboration between the European Commission, Companies and Associations from 8 industry sectors that work together to accelerate the development, validation and acceptance of alternative approaches to animal testing. In addition to 3Rs-related specific projects, the EPAA supports several dissemination and training activities that raise awareness and build confidence in new approach methodologies. Through the "3Rs Science" and "Refinement prizes", young scientists and laboratory technicians receive recognition and a financial award for important achievements. Each year, the EPAA also supports young scientists with outstanding work on alternatives to attend a high-profile scientific event and present their work. The student grants facilitate participation of young researchers in high-profile scientific conferences in Europe, such as ESTIV (European Society of Toxicology In Vitro), EUSAAT (European Society for Alternatives to Animal Testing), the World Congress on Alternatives and Animal Use in Life Sciences, EUROTOX or FELASA (Federation of European Laboratory Animal Science Associations). Building confidence in new approach methodologies among users from industry, academia and regulatory authorities is achieved through specific training activities supported by EPAA. Examples include two training videos on OECD accepted alternative methods for Eye Irritation (BCOP) and Phototoxicity (3T3 NRU) that have been developed earlier in collaboration with the Institute for In Vitro Sciences (IIVS). These videos are freely available via the EPAA website and YouTube, with subtitles available in several languages. In the area of skin sensitization, training and knowledge-sharing workshops on the use of alternatives in regulatory decision-making have been organized regularly by the EPAA. Some of these workshops have been hosted previously by the European Chemicals Agency, in Helsinki, and an EPAA-sponsored training is organised as satellite event to the WC11 in Maastricht.

**Presentation:** Oral



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## Reproductive toxicity and developmental toxicity addressed in REACH registration dossiers

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In 2011, ALTEX published a report on how reproductive and developmental toxicity were addressed in the first REACH deadline of 2010 (Rovida et al., 2011). The analysis of reproductive and developmental toxicity data in 400 records randomly selected in the ECHA database revealed a general lack of compliance with the provisions of the REACH regulation. A broad variety of existing data was used, and the read-across opportunity was very much exploited. Some new *in vivo* tests were performed in spite of the legal requirement to make a public proposal beforehand and wait for authorization by ECHA. *In vitro* tests were completely absent, even though there were many tests that could be applied to complement either read-across strategies or partially reliable existing data.

In 2016, ECHA started a massive control of the registration dossiers and it is now asking for many new *in vivo* tests to fix the problems that were already revealed in this analysis. Unfortunately, read across strategy to waive new *in vivo* tests is very often rejected, even if a proper justification would be possible and acceptable. This is an important message that deserves dissemination among the registrants.

An estimate of the number of animals that have been used until now will be also presented.

### Reference

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**Presentation:** Oral

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## A public private partnership facilitating development and uptake of 3R approaches

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As a public private initiative, the EPAA (European Partnership for Alternative Approaches to animal testing) is a voluntary collaboration between the European Commission, companies and European trade associations from seven industry sectors. The

partners are committed to pooling knowledge and resources to support and accelerate the development, validation and regulatory acceptance of alternative approaches. The overall aim is the replacement, reduction and refinement (3Rs) of animal use in regulatory testing.

The presentation will describe the set up and functioning of the EPAA and its activities, and it will provide examples of projects supported by the partnership. The examples relate to the areas of Skin sensitization and Carcinogenicity testing in agrochemicals.

The presentation will also point to the new EU institutional set up, highlight major upcoming events, and look at the next EPAA Action Programme.

To illustrate the EPAA's work four ongoing projects will be presented in detail by the project leaders later in this session: "New ideas for assessing systemic toxicity", "Harmonisation of 3Rs in Biologicals", "Optimal duration of non-clinical studies to assess the safety of monoclonal antibodies" and "Application of Physiologically-Based Kinetic Modelling in Safety Assessment".

For the EU, who has been pioneering progress in refining, reducing and replacing animal testing ever since animal welfare has been enshrined in the Treaties (EU, 2012), both within Europe and by using its strong international role as a regulator of markets, this is a priority partnership to foster mutual understanding and continued dialogue between scientists, regulators and industry.

### Reference

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**Presentation:** Oral

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## A human-derived proximal tubule-on-a-chip replicates ASO-induced kidney injury biomarkers

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Antisense oligonucleotides (ASOs) are an increasingly important therapeutic modality within the portfolio of the pharmaceutical industry. When tested in the clinic, SPC5001 ASO induced acute kidney injury (AKI). Preclinical mouse studies did not show any kidney-related safety concerns, suggesting human-derived *in vitro* models may be required. Therefore, we evaluated if SPC5001 induced toxicity and a clinically relevant biomarker



response in chip-cultured human-derived renal proximal tubule epithelial cells (HRPTEC). When chip-cultured HRPTEC were exposed to nephrotoxic ASO SPC5001 and safe ASO 556089 for 48 h, downregulation of their respective target mRNA, PCSK9 and MALAT1, was observed, referred to as productive uptake. Moreover, lactate dehydrogenase was released into the chip perfusate at the highest concentration (5  $\mu$ M) of SPC5001 ASO only, indicating cytotoxicity. Furthermore, kidney injury biomarkers were released into the chip perfusate upon continuous exposure to SPC5001 ASO for 20 days, including kidney injury molecule 1, neutrophil gelatinase-associated lipocalin, clusterin, osteopontin and vascular endothelial growth factor, each demonstrating unique time-dependent profiles. In conclusion, we have developed a human-derived proximal tubule-on-a-chip capable of replicating the release of injury biomarkers following exposure to a nephrotoxic ASO. Future validation using a larger set of ASOs will fully elucidate its translational value.

**Presentation:** Oral

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## Serum microRNA signatures as “liquid biopsies” for interrogating hepatotoxic mechanisms and liver pathogenesis

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Drug-induced liver-injury (DILI) is a leading cause of acute liver failure and the major reason for withdrawal of drugs from the market. Preclinical evaluation of drug candidates has failed to detect about 40% of potentially hepatotoxic compounds in humans. At the onset of liver injury in humans, currently used biomarkers have difficulty differentiating severe DILI from mild, and/or predict the outcome of injury for individual subjects. Therefore, new biomarker approaches for predicting and diagnosing DILI in humans are urgently needed. MicroRNAs (miRNAs) released into the peripheral circulation upon cellular injury have shown a promise as a new class of tissue-specific biomarkers. Using next generation sequencing, we examined profiles of serum circulating miRNAs in subjects with accidental acetaminophen overdose, hepatitis B infection, liver cirrhosis and type 2 diabetes as well as gender- and age-matched healthy subjects with no evidence of liver disease. We are first to show that different types of hepatic liver impairments feature distinct

signatures of circulating miRNAs and that this approach might be useful as minimally invasive diagnostic “liquid biopsies” enabling the interrogation of underlying molecular mechanisms of injury in distant tissues. Our study demonstrates for the first time that signatures of circulating miRNAs show specificity for liver injury phenotypes and, once validated, might become useful for diagnosis of organ pathologies as “liquid biopsies” for studying effects of drug candidates during clinical and preclinical drug development.

**Presentation:** Oral

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## The clinical translation of high-profile animal-based research reported in the UK national press

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Exaggeration of the significance of biomedical research discoveries and breakthroughs, and their translation to human benefit, are two important, contentious, and ongoing issues. These are of particular concern for animal research, which has considerable associated ethical costs. One consequence of such exaggeration in the media may be false public optimism and confidence in animal experiments. Prompted by these considerations, we examined reports of animal biomedical “breakthroughs” published in the UK national press in 1995 (20 years prior to the inception of this project), asking how many had resulted in approved interventions and human benefit. Media reports were identified in the “Nexis” media database (LexisNexis.com). To facilitate follow-up and reduce ambiguity, the inclusion criteria were that the intervention should be specific (e.g., named drug, gene, or biomedical pathway), and that there should be claims of clinical promise. Follow-up included examination of scientific literature databases, regulatory repositories, clinical trial databases, the World Health Organisation website, and other sources of scientific information. We found that the human relevance of animal studies is commonly over-stated in the media, prematurely implying often-imminent “breakthroughs” that rarely result in human benefit. This is consistent with exaggeration in institutional press releases, scientific papers, online news etc., and with increasing evidence of the generally poor clinical translation of animal research. It is imperative that more caution, honesty and objectivity is applied to reporting of animal data with regard to human relevance and benefit, and that the public views such news with greater caution. There are implications for the policies of governments, regulators, research funders, advocates and practitioners of animal research, and other stakeholders. Greater appreciation of the poor translation of animal experiments to humans



should result in a reappraisal of how animal research is approved and commissioned, with much more focus on alternatives, which are more human-relevant and humane.

**Presentation:** Oral

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## Sensitivity of mouse behavioral tests of anxiety to anxiolytic drugs approved for treatment of anxiety in humans: A systematic review

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Anxiety disorders are amongst the most common mental health conditions, requiring still new and better treatments. Animal models play an important role in the preclinical phase of drug development, as new drugs require testing to ensure safety and efficacy. However, the sensitivity of widely used behavioral tests for anxiety has been questioned, as they often fail to replicate the same results in different model organisms or with different anxiolytic compounds. We identify here a potential cause for a deficient animal to human translation and a wasteful use of animal lives. Thus, we investigated the sensitivity of outcome measures of mouse behavioral tests of anxiety to anxiolytic drugs approved for the treatment of anxiety in humans. We conducted a quantitative systematic review of research papers that tested anxiolytic drugs used in humans against a control treatment using the most common behavioral tests for anxiety in mice. PubMed and EMBASE databases were searched on 21.8.2019 for studies published in English and 822 papers were identified for inclusion. Reported effects and effect sizes for specific compounds varied widely between studies. Meta-analyses were performed on size of treatment effects on outcome measures, as standardized mean differences (Hedges' *g*). Overall, we found weak or no effects of most anxiolytic drugs on a wide range of outcome measures, questioning the sensitivity of these tests to a broad range of anxiolytic drugs, other than benzodiazepines. Additionally, we found high heterogeneity (*I*<sup>2</sup>) across most outcome measures and assessed an overall high risk of bias. Our results call for further investigation of the construct validity and predictive validity of preclinical tests of anxiety in view of more responsible research. Thus, better preclinical tests will improve translational power, while reducing the use of animals for inconclusive research.

**Presentation:** Oral

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## *In-vitro* inhalation experimentation design and dosimetry considerations for *in-vitro* to *in-vivo* prediction of respiratory toxicity

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Investigating biological effects upon inhalation has its application in pharmaceutical research and toxicology. State-of-the-art biological models include a wide range from cell-free approaches over single cell lines to more complex models such as 3D-systems and *ex vivo* models such as precision cut lung slices (PCLS) or the isolated perfused lung (IPL). Especially air-liquid interface (ALI) culture models have come into focus during the last years due to relevance, efficiency and versatility. However, to provide relevant data for *in vitro* to *in vivo* prediction of effects, the whole experimental concept has to be aligned to this objective including generation of test aerosols, consideration of adequate controls, definition of reference substances and characterization of the test setup. Based on inclusion of these aspects and careful dosimetry considerations, meaningful *in vivo* prediction may result. As an example, an *in vitro* inhalation model for testing dry powder fungicide aerosols is presented. It includes generation of highly concentrated aerosols from small amounts of powders, exposure of a human lung alveolar epithelial ALI cell model (A549) in an optimized exposure device (P.R.I.T.<sup>®</sup> ExpoCube<sup>®</sup>), determination of cytotoxicity and dosimetry considerations. Whereas the focus of this model is local acute toxicity in the lung, physiological effects were investigated in parallel in an IPL approach enabling investigation of physiological effects related to breathing mechanics, such as reduction of tidal volume or dynamic lung compliance and the relative increase in lung weight. Correlation of the *in vitro* and *ex vivo* derived dose values to literature data from acute *in vivo* animal inhalation studies showed very promising prediction of LD50 values and respiratory effects.

As a result, it is concluded, that – based on adequate experimental concepts – present alternative models are able to deliver substantial data for prediction of respiratory effects from inhalation of airborne substances, such as gases, vapors or aerosols.

**Presentation:** Oral

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## Update on innovative approaches to share organs and tissues in science

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The reduction of lab animal usage can be achieved by, e.g., improved statistical and experimental planning, in-depth pre-analysis in suitable cell culture models or conscious handling of biological resources and materials that are left during the animal experiments. Therefore, sharing of organs and tissues of animals sacrificed for scientific purpose, surplus animals or those used for organ collection under anesthesia or for educational purposes provides the great opportunity to sustainably reduce the animal number. This has also been taken into account by the European directive 2010/63/EU, which includes the request that “Member States shall facilitate, where appropriate, the establishment of programs for the sharing of organs and tissues of animals killed.” (Recital 27 and Article 18 2010/63/EU). Therefore, several small initiatives can be identified in Europe which are mostly focusing on biobanking of one specific animal species (EU PRIMNET, MIDY), tissue (NERD) or disease model (SEARCH-Breast, SharmUK). A more general approach was launched under the name AniMatch (<http://www.animatch.eu>) – an innovative web-based platform that allows scientists to register and publish or search for offers to facilitate the multiple use of freshly killed animals. The overall number of scientists and animal welfare bodies is convinced of the resource-sparing and morally sustainable approach of those initiatives. Nevertheless, challenges are seen in the additional effort needed as well as in the lack of incentives which need to be addressed and discussed. Within the presentation, the goals, advantages and limitations of those initiatives will be presented in accordance with a current update on acceptance and challenges within the scientific community. We will also highlight experiences from the use of AniMatch in Switzerland and discuss options for progress. Besides the moral exculpation for scientists, sharing initiatives provide a cost-efficient way to use existing infrastructure and to conserve resources in accordance with reducing lab animal usage.

**Presentation:** Oral

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## Engineering a dynamic model of the alveolar interface for the study of aerosol deposition

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To study the complex interaction between inhaled particles and lung tissue, both *in vitro* and *in vivo* models can be used. However, traditional *in vitro* models cannot reproduce the entire complexity of the alveolar environment, while *in vivo* models do not consider interspecies differences, are expensive and pose ethical problems. To overcome these issues, an innovative bioreactor was developed. This system, named DALI (Dynamic model for ALveolar Interface), consists of an aerosol generator and a bioreactor with a moving membrane placed between an air-liquid interface to simulate physiological lung muscle stretching. An electrospun membrane made of Bionate<sup>®</sup> was selected as support of the alveolar barrier, since it was shown to be porous, biocompatible, cell adhesive and highly elastic, allowing for cyclic stretching of the membrane. To mimic natural breathing, an external compressed air system was used to stretch the elastic membrane. Finally, a Quartz Crystal Microbalance was integrated to quantify the aerosolized nanoparticles on the cell layer.

The bioreactor was validated in terms of liquid and air tightness, biocompatibility, and capability of applying a cyclic strain. Preliminary biological studies were performed to investigate the effects of flow and mechanical stretch on the cells. Human alveolar epithelial cells were cultivated within the bioreactor under fluidic dynamic conditions, applying a cyclic mechanical strain on the cells (5% linear strain, corresponding to the normal breathing (Felder et al., 2019)). Biological experiments suggested the suitability of the system for the culture under flow. Further studies will be performed to optimize cell culture under stretch conditions.

To conclude, the system could help in bridging the gap between *in vivo* and *in vitro* models, overcoming some of the shortcomings of traditional *in vitro* models.

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**Presentation:** Oral

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## The human-based *in vitro* 3D arthritic joint model for preclinical drug testing

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Our ultimate goal is to study the influence of potential drug candidates in an experimental setting of arthritis. Although a variety of models for RA already exists, they either employ phylogenetically distant species or rely on human cells cultured in an oversimplified environment and are not able to reflect the human disease in all aspects. Hence, we developed a complex human *in vitro* 3D joint model to study the influence of the main cytokines involved in the inflammatory response of RA and the potential of therapeutic treatment.

Well characterized human bone marrow-derived mesenchymal stromal cells (hMSCs) were used to develop a 3D bone and a 3D cartilage component. The 3D bone model was developed by successfully seeding hMSCs on a  $\beta$ -TCP scaffold. Osteogenic differentiation was verified demonstrating an increase in mineralized bone volume and the induction of bone-related gene expression. Chondrogenic phenotype was verified by Alcian Blue staining and by the reduced expression of COL1A1 and abundant expression of COL2A1 on protein level. Co-cultivation of both components leads to formation of an interconnection between the two 3D components. Cytokine stimulation induces the upregulation of matrix-degrading metalloproteases particularly in the cartilage component. In addition, a cytokine-induced upregulation of the inflammation markers IL8 and TNF, the metabolic marker LDHA and the angiogenic marker VEGF can be observed in bone and cartilage. All these cytokine-mediated effects are antagonized with a cocktail of specific immunomodulatory drugs. Modeling the synovial membrane, we successfully created a confluent hMSC cell layer. In response to cytokine stimulation, the synovial membrane showed an upregulation of IL6, HIF and MMP13 expression on mRNA level.

Our data provide evidence that our human 3D joint model mimics the main features of human RA and may serve as a pre-clinical tool for the evaluation of anti-inflammatory drugs in a pathophysiological human setup.

**Presentation:** Oral

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## Clicker/target training of research animals as a refinement

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The use of clicker or target training is well established among animal behaviorists and trainers working with pet animals, especially dogs. However, these methods of training are less commonly used with the diversity of research animals at our institutions. The majority of reports regarding the use of positive reinforcement training (PRT) with research animals refer to nonhuman primates, dogs and swine. Through the use of PRT, these animals are trained to cooperate with a variety of common procedures, such as weighing, blood sampling, urine collection, etc. However, there are numerous opportunities to broaden the spectrum of animals that can be trained to voluntarily engage in a task, as well as the goal of the PRT. This presentation will describe the principles of clicker and target training as methods of PRT, as well as a successful approach to implement this type of training program at your institution. Using the cat as an example, a method for promoting well-socialized animals and to aid group housing strategies will be described. The value of including PRT as a husbandry and experimental Refinement will be discussed.

**Presentation:** Oral

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## Looking back at 5 years providing 3Rs training in Europe: An SME perspective

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Existing 3Rs Education and Training (E&T) courses cover animal research and animal testing. When it comes to alternative test methods, it is well admitted that validation studies and regulatory acceptance of alternative test methods are a long process but not the end of the journey to become part of routine use of the

new technologies. Multiple initiatives exist to facilitate and improve the confidence of end-users, be a regulator, be a test provider or be a test performer. This diversity of end-users complexifies the needs and expectation for 3Rs E&T. Therefore, specific solutions need to be provided accordingly. Most of the 3Rs E&T formats are already covered with theory thanks to webinar, massive open online course or lectures at university and practice with hands-on training or case studies. However, some gaps always exist since it is almost impossible to be at the same pace as technology evolves. Currently most of the recognition and accreditation of such 3Rs E&T are mainly centralized by scientific societies and universities. Therefore, there is limited opportunity for private sector and NGOs to feed in curricula in this field in spite of the multitude of initiatives and efforts. This talk intends to describe some of the accreditation mechanism and discuss ways to improve the effectiveness and recognition of 3Rs E&T courses coming from other sectors.

**Presentation:** Oral

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## Gender inequality in science – Finding the end of the rainbow

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“The story of women’s struggle for equality belongs to no single feminist nor to any one organization but to the collective efforts of all who care about human rights.” (Gloria Steinem, world-renowned feminist, journalist and activist.) Thus, gender inequality and missing diversity are major issues that we need to address together as community. Although the scientific community is recently taking action and stimulating discussions, the problem persists. Women in science, technology, engineering, and mathematics (STEM) mainly face challenges regarding gender bias in publications and third-party funding, pay disparity and harassment at the workplace (Huang et al., 2020; Raymond et al., 2019). In addition, it has been reported that women are underrepresented in PhD programs especially focusing on maths and engineering. However, sexual identity can also be a reason for unfair treatment and discrimination that seems to be more pronounced in science than other areas. Hence, it is obvious that one needs to find ways to promote recognition of gender intersectionality, equity, and inclusivity of all gender and LGBT+ in academia (Gibney, 2019). Diversity benefits science and should be acknowledged as such.

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**Presentation:** Oral

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## Developing an *in vitro* model of glucocorticoid-induced osteoporosis

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The healthy bone homeostasis is characterized by a balanced, dynamic and continuous remodeling process based on specialized cells such as osteoblasts, osteocytes and osteoclasts (Scheinpflug et al., 2018). Glucocorticoids (GCs) are commonly used to treat patients with inflammatory diseases. However, long-term treatment with GCs can potentially lead to several adverse effects such as the inhibition of osteoblast proliferation and the increase of osteoclastic activity resulting in osteoporosis (Buttgerit, 2020). Hence, the aim of our project is to i) develop an *in vitro* trabecular human bone model, ii) integrate this bone model into a perfusion system to accelerate mineralization and provide biomechanical stimuli and iii) applying prednisolone to induce osteoporosis. Here we present our initial results describing the successful differentiation of osteoblasts and osteoclasts in a 3D environment, and the accomplished integration of the bone model into a perfusion system. In a first step, different cultivation conditions were tested to allow optimal osteogenic or osteoclastic differentiation. To this end, a) human bone marrow derived mesenchymal stromal cells (hMSCs) were treated with osteogenic medium, and b) peripheral blood-derived monocytes were differentiated into osteoclasts by supplementation of M-CSF/RANKL. Calcification of hMSCs was evaluated via Alizarin-red staining. Osteoclasts were identified by immunofluorescence staining as multinucleated (DAPI) giant F-actin ring forming cells owing TRAP and Cathepsin K activity. Additional gene expression analyses confirmed the up-regulation of osteoclast-specific genes. In parallel to the monolayer cultures, cells were successfully transferred on  $\beta$ -tricalcium phosphate – a suitable bony-like scaffold. Furthermore, first experiments in a dynamic bioreactor platform (OSPIN GmH) were conducted to evaluate the influence of shear stress on the cells and model systems. By combining several cell types, a suitable scaffold and biome-



chanical stimuli (perfusion), we aim to provide a valid testing platform to study underlying disease mechanism and for drug development.

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**Presentation:** Oral

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## Combining *in vitro* and *in silico* modelling to study cytokine-driven cartilage degradation

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The importance of articular hyaline cartilage becomes clear when cartilage is destroyed such as in osteoarthritis (OA). Cartilage destruction is accompanied by a tremendous loss of life quality due to deprivation of agility and flexibility, morning stiffness, and pain during every step. Understanding the pathophysiological processes of cartilage degradation requires adequate model systems to develop new therapeutic strategies. Our project aimed at computing an *in silico* model based on the results of our recently developed *in vitro* OA model to enhance validity and translatability towards a more sophisticated simulation of OA. In detail, the used 3D *in vitro* model was based on 3D chondrogenic constructs generated solely from human bone marrow derived mesenchymal stromal cells (hMSCs). Our chondrogenic *in vitro* model revealed the expression of the cartilage specific markers collagen 2 (Col II) and aggrecan and the deposition of glycosaminoglycans in the extracellular matrix. The cell concentration was slightly decreased over 3 weeks of untreated cultivation. After stimulation with pro-inflammatory cytokines, the constructs showed an increased expression of inflammatory markers (IL-1, -6 and -8) and matrix degrading enzymes (matrix metalloproteinase-1, -3 and -10) compared to untreated controls. During histological and histomorphometric analysis, we observed a decreased compactness of extracellular matrix, a loss of Col II, a significantly reduced cell concentration, and altered cell morphology. As a subsequent mathematical strategy, we described the biological processes by differen-

tial equations considering, e.g., the change in cell numbers and Col II concentrations in different areas of the constructs to include spatial resolution over time. By combining methods used in biological research and those used in mathematical systems biology, we aim at developing a valid, efficient and attractive alternative approach to test possible treatments for OA, examine underlying mechanism of OA and cartilage repair to further support translation in OA research.

**Presentation:** Oral

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## Application of *in silico* models to support decision making in toxicology

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Chemical toxicity has traditionally been assessed using *in vivo* tests to ensure human safety. However, animal testing is generally disfavoured by both regulatory bodies as well as the public, and the human relevance of data from such animal models is debatable. Consequently, *in silico* models are becoming an increasingly important tool to support decision-making within toxicology, but knowledge of how to assess and apply such models remains critically important if they are to be used effectively. Whilst the OECD principles for the regulatory use of (Q)SAR models provide a useful starting point (especially for model developers) these alone are not sufficient to guide a user to a safe, supported and confident decision (OECD, 2014). This talk will describe:

- (1) how positive and negative predictions from classical *in silico* models (expert knowledge-based and statistical-based systems) can be used within ICH M7 to predict *in vitro* mutagenicity; and in combination with *in vitro* assays to predict skin sensitisation hazard and potency.
- (2) how to leverage machine learning and artificial intelligence (AI) to develop a secondary pharmacology model which can learn from multiple proprietary datasets without disclosure of confidential structures (federated learning).
- (3) how to use an expert knowledge-based system to replace a human expert and update dermal sensitisation thresholds (DSTs) used in quantitative risk assessment (QRA).
- (4) how to explore toxicity by linking existing knowledge and assay data to adverse outcome pathways (AOPs).

These examples will illustrate how *in silico* models are applied within toxicology, including how to assess the confidence in each prediction, and how to use supporting information as evidence that allows a model to be challenged.

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**Presentation:** Oral

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## Criterion guided interviews to validate competence in designing animal experiments

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According to Directive 2010/63/EU, we have to ensure competence levels of personnel involved in animal experiments including those “designing procedures and projects”. The importance of this is emphasized by observations that many research findings within animal research are questionable due to poor methodology (Macleod et al., 2015). In order to improve the quality, guidelines like PREPARE (Smith et al., 2017) and ARRIVE have been introduced. Adhering to these guidelines requires thorough knowledge of statistics, animal or other models and of potential bias risks. All of these can be specific for different experiments. It is important to establish how well researchers can comply with the guidelines when designing experiments.

We considered that assessment methods should be available independent of training and as an aspect of lifelong learning in animal science (Dutch CPD document, 2018). Moreover, establishing competences on the job is a prerequisite.

For this purpose, we designed an assessment consisting of a criterion guided interview (CGI) and a rubric that addresses relevant learning outcomes, that fulfil education requirements under the Directive (Working document, 2014). Some were revised to ensure hands-on application of these outcomes. This includes responding to the challenges within the current research culture.

During the CGI the work protocol is used as a “portfolio” to assure relevance in daily practice. The assessment is continuously validated within the field, using a network of principle investigators as co-assessor. To ensure validity and reliability, assessors have been trained.

In a first trial, we experienced that a CGI not only allows to identify competence, but also has enormous potential to help the participant to develop further (assessment for learning). It can also be used as a formative learning tool earlier in the training and is appreciated as such during the final assessment. To see an example CGI go to <https://youtu.be/UP2vEbJMnPI>.

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**Presentation:** Oral

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## Replacing fetal bovine serum (FBS) – Innovative alternatives and transition strategies

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Fetal bovine serum (FBS) has been used for decades as a supplement in a wide range of cell culture applications in cell-based research, drug discovery, diagnostics, toxicity testing, cell therapy, *in vitro* fertilization, human and animal vaccine production, and biopharmaceutical manufacturing. FBS has been considered rather unique in its universal capacity to support the growth of multiple cell types in culture (van der Valk et al., 2018). Although widely used, there are various reasons to find suitable alternatives to the use of FBS: amongst them the ill-defined nature, animal welfare considerations, and economic issues, sourcing and supply, xenogenic properties and pathogen safety issues. Still, FBS is the most widely used growth factor supplement for the expansion of human cell therapy products (Bieback et al., 2019). However, there has been a strong impetus from regulatory agencies and biomedical professionals in the field to develop methods for cell expansion that do not utilize animal products in their production process. Human platelet lysate (hPL) has been identified as a possible growth supplement contender – rich in growth factors and produced in most cases from expired platelets (PLTs) – that can be used to replace FBS. hPL is essentially the product of lysing human PLTs typically involving freezing and thawing to release trophogens and growth factors. Especially in protocols to manufacture mesenchymal stem/stromal cells (MSCs) for clinical



use, hPL has been established as a safe substitute to FBS (Bieback, 2013). Still issues remain, such as ill-defined nature and economic issues. In terms of standardization, chemically defined media appear as the ultimate achievement. Since these media need to maintain all key cellular and therapy-relevant features of the target cells, the development of chemically defined media is still – albeit highly investigated – only in its beginning.

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**Presentation:** Oral

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## “My Animal Research: Experimental Design”: A personalized, practice-based learning track for PhD students

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There is a growing concern about the poor quality of animal research, based on observations that many research findings within animal research are questionable due to poor methodology (Macleod et al., 2015). With the ambition to improve the quality of animal research, Utrecht University, University Medical Center Utrecht have joined forces. They are contributing to the change of the current research culture.

As a first step towards this ambition, we developed an internationally oriented learning track for PhD students in animal research, using Educational Design Research (EDR) as a methodological framework (Plomp, 2013). Based on the educational concepts of connectivism (Siemens, 2005), personalized learning and workplace-based learning, the learning track consists of several online and face-to-face learning activities (i.e., blended learning). It focusses on the competence of “designing procedures and projects” (Working document, 2014), with the aim to deliver an executable work protocol at the end. Key components of the learning track are a diagnostic assessment with personalized feedback at the start, a free to use online knowledge data-

base, working sessions with experts, reflection and culture interventions, and a personalized final assessment.

The pilot demonstrated great potential in creating awareness of the importance of good experimental design, well-motivated choice of (animal) models and a good statistical plan and increases the knowledge on these topics. More importantly however, the setup allows for direct implementation of the acquired knowledge. Further development could be directed towards better preparation towards the flipped classroom approach, intensifying training in statistics, and further integration of the culture sessions. This will strengthen the effect on participants’ experimental designs and overall quality of future animal experiments. Additionally, also due to the assessment and certification, it will establish “designing procedures and projects” as a validated competence.

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**Presentation:** Oral

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## Mapping potential biomarkers for early sporadic Alzheimer’s: Status of the Interreg VL-NL project “Memories”

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Alzheimer’s disease (AD) is a common neurodegenerative disorder characterized by progressive memory loss with an increased prevalence. Although up to 5% of all AD cases can be assigned to specific gene mutations (familial AD, fAD), the vast majority of AD cases is sporadic (sAD). This strongly suggests that environmental factors may contribute to the development



of AD. Emerging evidence suggests that exposure to exogenous chemicals including heavy metals, pesticides and air pollutants may contribute to AD. In the Interreg-EU project “Memories” (<http://www.herinneringen.eu>) we used different bioinformatics approaches to analyze sequencing data from AD-related iPSC cortical neuron models as well as human tissue (blood plasma, cerebrospinal fluid (CSF), tissue of different human brain areas) with the aim to develop new biomarkers, e.g., miRNA profiles as early diagnostic tools of sAD. MiRNAs are small non-coding RNA molecules that influence gene regulation of essential biological pathways. The primary goals of this project is to develop a specific biomarker for sAD based upon profiles of blood plasma circulating miRNA (van den Berg et al., 2020). Secondly, by comparing these profiles with miRNA and mRNA profiles obtained from patients and iPSC-derived cortical neuronal models, a better interpretation of the biological mechanisms involved in the development of sAD. We used sequencing data from mRNA and miRNA expression profiles from iPSC-derived human cortical neuron models exposed to various environmental chemicals. Data of changes in m(i)RNA expression were also be obtained from tissue of different brain areas, and from circulating miRNA (cmRNA) profiles in cerebrospinal fluid (CSF) and blood plasma of AD patients. We investigated if profiles of cmRNA in blood can be used as a diagnostic tool in by reflecting initiation and progression of Alzheimer’s disease and can be correlated with miRNA profiles in different tissues and in specific affected areas of the brain.

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**Presentation:** Oral

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## Reducing, replacing and refining aquatic vertebrate testing in the identification of endocrine disruptors

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Regional regulations are starting to explicitly require that endocrine disrupting properties be investigated as part of the safety assessment process (EFSA/ECHA, 2018). This can include conducting new vertebrate tests for substances already on the market. Different jurisdictions are utilizing different approaches. However, all share common themes of testing for endocrine activity and adverse effects. Testing for activity usually involves *in vitro* and *in vivo* assays on selected endocrine pathways. For ecotoxicological evaluation, these assays can be performed across various animal species including mammals, amphibians and fish. Results indi-

cating activity (i.e., that an endocrine mechanism may be present) in these initial tests usually trigger further higher tier *in vivo* assays. These provide data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. Higher tier assays are very animal and resource-intensive and technically challenging to conduct. The identification of endocrine disrupting properties is an area which could use large numbers of animals (Lagadic et al., 2019), contradicting stipulations set out within many regulatory frameworks that animal studies be conducted as a last resort.

In February 2020, the NC3Rs in collaboration with HESI hosted a workshop “Investigating endocrine disrupting properties in fish and amphibians: Opportunities to apply the 3Rs”. Over 50 delegates attended from North America and Europe, across academia, consultancies, regulatory agencies and industry (contract research organizations, agrochemicals, industrial chemicals, consumer products and pharmaceuticals). They discussed the challenges and opportunities in applying refinement and reduction approaches within the current mandatory animal tests, and in more widely utilizing replacement approaches – including *in silico*, *in vitro* and embryo models. Steps needed to enable the application of 3Rs approaches in practice were also identified. This presentation provides an overview of the workshop discussions.

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**Presentation:** Oral

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## Smart use of social media in 3Rs

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For better or for worse, society has been transformed by social media (Britton et al., 2019). Twitter – a social microblogging platform created in 2006 – claims 321 million active users in 2018. Approximately 500 million tweets are tweeted each day. Thanks to the development of a specific twitter application, more than 700,000 tweets were collected from October 2014 to March 2020 based on the following hashtags: #animaltesting OR #animalfreetesting OR #animalfreetests OR #animalexperiments OR #3Rs OR #3R OR #BeCrueltyFree OR #endanimaltesting OR #stopanimaltesting OR #stopvivisection. Based on this unprece-



dented analysis, the authors were able to 1) identify the absolute number of users as well as its variation over the last seven years, 2) the popular tweets 3) extracting new hashtags from pre-defined ones 4) differentiate users that are “preaching to the choir” or “singing from the rooftop” (Côté and Darling, 2019). Moreover, a Twitter sentiment analysis in R-language was performed resulting in classification emotion content of the tweets ranging from anger to surprise or joy with a majority of fear. Furthermore, elasticsearch was now included to further refine the database analysis. Thanks to this weight of evidence, the author argues that by communicating on social media, peers are able to interact further and revolutionize the way science is shared and spread (Glausiusz, 2019).

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**Presentation:** Oral

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## Data access and EU institutions

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ECHA (European Chemicals Agency), EFSA (European Food Safety Authority) and EMA (European Medicines Agency) are related to the Committee on Environment, Public Health and Food Safety (ENVI) at the European Parliament (EP). All three agencies play an important role in collecting safety data for all manufactured goods that are introduced to the EU market. In all sectors represented by the agencies, similar types of data (toxicological endpoints) are collected, but they differ in format, transparency and confidentiality level assigned to them. Such differences are not due to legal requirements, but they are mostly linked to EU Agency internal policies. The lack of harmonization has dire consequences for the implementation costs of EU regulations, for the performance of the different industry sectors and for the excessive/redundant use of animals for safety testing. Moreover, the efficacy of the agencies themselves is reduced. This oral presentation intends to provide an overview of the current big data initiatives (e.g., OpenFoodTox, <https://data.europa.eu/euodp/data/dataset/openfoodtoxefsa-s-chemical-hazards-database>) at the EU agency level, collaborative activities at stakeholder level (e.g., AMBIT; [<https://bd4bo.org/>\) or at European Commission level \(e.g., DG ENV, 2019/1010/EU\). Last but not least the author will describe the adoption of a pilot project funded by the EP on “Feasibility on a common open platform on chemical safety data, <https://etendering.ted.europa.eu/cft/cft-display.html?cftId=5516>” currently led by the European Commission. The goals of the pilot project are to facilitate seamless sharing of data between authorities and provide public access to researchers, regulators, industry and the citizen at large. This will promote: a\) transparency and trust in EU decision making, b\) research and data analytics, c\) innovation d\) less animal testing & more predictive toxicology, and e\) better regulatory decision making and informed consumer choices.](http://cefic-iri.org/toolbox/am-</a></p>
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**Presentation:** Oral

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## Exposure based safety assessment of cosmetics

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The magnitude, duration, frequency, and route of exposure can all dramatically influence the toxicity exhibited by a compound. The ability to fully understand in-use scenarios and the subsequent local and systemic exposure metrics can inform the degree of hazard data needed to support a risk (exposure x hazard) based approach. Tiered approaches are utilized for the exposure assessment and initially rely on conservative worst-case assumptions (e.g., 100% absorption) which can be refined if needed. In some cases, exposure-based waiving using thresholds of toxicological concern (TTC) may be sufficient to assess the safety of materials with very low exposure. Higher exposures may require an understanding of the amount of ingredient that can get into the body and this information can then be used for the human safety assessment as well as inform the dose range for *in vitro* assays. Experimental (e.g., skin penetration) and computation approaches (e.g., physiologically based kinetic (PBK) modelling) are important tools for increasing understanding of exposure in a tiered approach. Characterizing the degree of uncertainty at each stage is important and is considered when assessing if the data is suitable for the purpose or if further studies are required. The current presentation will discuss tiered approaches to exposure assessment, both local and systemic, and how such knowledge can be used in a human safety risk assessment and to guide relevant doses for *in vitro* hazard assessment. Example exposure assessments will be provided for single chemical exposures as well as product exposures.

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## Humane end-points tailor made

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With the term “humane end-point” appearing in the Directive 2010/63/EU Member States are required to implement the use. Humane end-points are traditionally defined as, “the earliest indicator in an animal experiment of severe pain, severe distress, suffering or impending death”. However, with a strong movement to reduce severe suffering in animal experimentation we advocate a more recent definition for humane end-points (Hendriksen et al., 2010) as “a refinement strategy during the experimental design phase to ensure minimized pain, suffering or distress experienced by animals during an experiment”. With this use general operating procedures or guidelines for a research field are less applicable since humane end-points are per definition part of the experimental design of a singular experiment.

In practice this means that the humane end-points are reviewed in light of the specific research question, procedures, animals used and critical phases in the study. This results in more tailor made end-points that ensure minimizing suffering while keeping maximal scientific benefit. With a focus on pre-clinical end-points in practice this means that welfare evaluation is not a standard procedure. All animals are monitored in line with the design specific end-points at hand.

This review and labor intensive process in monitoring the animals does yield that procedures prospectively classified as mild to never reach the severe category because humane end-points are set in the moderate category. An example of a tailor made humane endpoint considering tumor size is keeping the tumor burden limited to the minimum required for a valid scientific outcome (Workman et al., 2010). Practice shows that a reduction of the traditional maximum tumor size is often possible when considering science and design.

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**Presentation:** Oral

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## An industry perspective on strategies for integrating new approach methodologies for next generation risk assessment: Coumarin as a case study

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Several theoretical frameworks describing a tiered approach for NGRA have been published over the past few years (Berggren et al., 2017; Dent et al., 2018), but concrete next generation risk assessment (NGRA) examples of how to analyze, integrate and interpret all the data obtained from new approach methodologies (NAMs) in order to inform a safety decision are still lacking.

In this work, we set out to integrate currently available NAMs in a hypothetical safety assessment of 0.1% coumarin in face cream and body lotion to make a safety decision without using any *in vivo* animal data (Baltazar, submitted). Internal concentrations (plasma C<sub>max</sub>) were estimated using a physiologically-based kinetic (PBK) model for dermally applied coumarin (Moxon et al., 2020). Biomarkers were selected to provide evidence of whether coumarin may cause specific cellular effects (Eurofins Safety44™ screen and BioMap® Diversity 8 Panel) or non-specific effects (*in vitro* cell stress panel (Hatherell et al., submitted) and high-throughput transcriptomics). In addition, *in silico* alerts for genotoxicity were followed up with the ToxTracker® tool.

The PoDs from the *in vitro* assays were compared to exposure estimates (plasma C<sub>max</sub>) to calculate a margin of safety (MoS) distribution which is used in the risk assessment decision. The predicted C<sub>max</sub> values for face cream and body lotion were lower than all PoDs with a MoS higher than 100. Furthermore, we can conclude that coumarin is not genotoxic, does not bind to any of the 44 targets and does not show any immunomodulatory effects at relevant exposures.

The continued development and application of NAMs in a decision-making context will play an increasing role in fulfilling the ambition to assure safety of novel ingredients without the



need for any animal testing, but confidence in NAMs will only come with learning by doing and sharing more case studies.

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**Presentation:** Oral

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## Phenotypic high throughput screening in a human iPSC-derived hepatocyte model of steatosis reveals inhibitors of ER-stress induced lipid accumulation

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Liver fat deposition characterizes non-alcoholic fatty liver disease (NAFLD) which, when associated with endoplasmic reticulum (ER), autophagic, and oxidative stresses and other hepatocellular damage, can progress to non-alcoholic steatohepatitis (NASH). NAFLD is a growing worldwide health issue affecting one-third of the population including 75-92% of the obese population of which 37% progress to NASH. There are currently no drugs approved for the treatment of NAFLD/NASH and a lack of pre-clinical models of disease. *In vitro* models that better predict a drug response with a particular mechanism of action related to the disease signature that can be used for discovery of a small molecule that decreases hepatic fat and alleviates associated cellular stresses would represent a major breakthrough in human therapeutics for treatment of severe NAFLD/NASH. We have reported on phenotypic ER stress-induced TAG accumulation assay in iPSC-Hep which recaptures several metabolic changes characteristic of steatosis associated with NAFLD (Parafati et al.,

2018). To extend our model, we have screened an open sourced library from Astra Zeneca consisting of 13,000 well bio-annotated chemical leads with maximal coverage of target activities. Hit analysis revealed cyclin dependent kinases (CDK2/4) inhibitors regulating the C/EBPalpha pathway is driving reversal of the phenotypic effect. CDK2/4 protein levels are shown to be elevated in patients with fatty livers (Jin et al., 2016). These results, to be presented, provide a proof of concept that hiPSC-derived hepatocytes are amenable to high-throughput screening and exhibit sufficient metabolic function to be used for drug efficacy studies for NAFLD.

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**Presentation:** Oral

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## Metabolic maturation of human iPSC cell-derived hepatocyte-like cells in multicellular spheroid culture

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Human iPSC cell-derived hepatocyte-like cells (hiPSC-HLCs) is now commercially available, but their metabolic functions are still not comparable to real hepatocytes. Three-dimensional (3D) multicellular spheroid culture is well known as a technique for enhancing liver functions in primary cells as well as liver cancer cell lines. hiPSC-HLCs, however, have relatively low aggregation property, it is difficult to stably form spheroids by conventional methods. The objective of this study is to confirm whether hiPSC-HLCs can be efficiently converted into spheroids and exhibit metabolic maturation as hepatocytes. ReproHepato (ReproCELL, Yokohama, Japan) was used as commercially available hiPSC-HLCs. In order to forcibly aggregate hiPSC-HLCs, we applied a method using 3% methylcellulose (MC) medium (Kojima et al., 2012). After thawing a frozen vial, hiPSC-HLCs were suspended in a culture medium at  $2 \times 10^6$  cells/mL, and 1  $\mu$ L of the cell suspension was injected into 3% MC medium. The 2000 cells gathered in 10-30 min and formed multicellular spheroid in 24 h. The spheroid was able to be removed from the MC medium without collapse and stably cultured in an ultra-low attachment 12-well plate at least 2 weeks. We then examined the expression of CYP3A4, CYP2B6 and CYP1A2 genes. After culturing for 6 days from the injection, the expression levels of these enzyme genes were almost the same as those in the two-dimensional control culture. By culturing for 14 days, the expression of the genes was improved several hundred times. The time-course observa-



tion showed that the expression level of the genes gradually increased from day 6. Other CYP genes, conjugation enzymes, and transporters were also induced by the 3D culture. Besides, genes for amino acid, sugar, and urea metabolizing were upregulated in the spheroid culture condition. These results indicate that formation of multicellular spheroids play a pivotal role for metabolic maturation of hiPSC-HLCs.

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**Presentation:** Oral

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## Evaluation of a new approach methodology toolbox for the next generation risk assessment of systemic toxicity

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Over recent years a number of New Approach Methodologies (NAMs) have been developed to evaluate the bioactivity of chemicals within a Next Generation Risk Assessment (NGRA) context. NGRA is an exposure-led hypothesis-driven approach that works to integrate New Approach Methodologies (NAMs) into a safety decision for consumers. There is a need to conduct and share case studies of *ab initio* NGRA to build confidence in the ability to use only NAMs in risk assessment. A forthcoming publication presents the risk assessment of 0.1% coumarin in a face cream and body lotion using *in vitro* assays to determine consumer safety (Baltazar et al., submitted), however the applicability of these techniques for other compounds needs to be assessed to give confidence in this approach moving forward.

This work focuses on an approach to evaluating three NAMs (high throughput transcriptomics, *in vitro* cellular stress (Hatherell et al., submitted) and the Eurofins Safety44<sup>®</sup> Screen) with respect to their use in systemic toxicity risk assessments. These assays, together with approaches to estimate the relevant human exposure, such as physiologically-based kinetics modelling, skin penetration and free concentration work, could form a core toolbox within an NGRA framework and provide *in vitro* points of departure for calculation of a margin of safety. To build confidence in the use of this toolbox, data will be generated for 30-50 compounds with identifiable human use scenari-

os, and the safety decisions based on *in vitro* points of departure compared to those using more traditional risk assessment approaches. These data will be used to develop a statistical modelling framework for characterising uncertainties in the margin of safety distributions, which can then be used as part of an expert-based assessment of an appropriate MoS for a given risk assessment.

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**Presentation:** Oral

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## Qualification of cardiac microphysiological models for drug screening

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Animal models are 78% accurate in determining whether drugs will alter contractility of the human heart. Cardiomyocytes from human induced pluripotent stem cells (hiPSC-CMs) are increasingly recognized as valuable for determining the effects of drugs on ion channels but do not always accurately predict contractile responses of the human heart. This is in part attributable to their immaturity but sensitivities of measurement tools may also be limiting. Here, we benchmarked our hiPSC-CM based microphysiological models through a blinded drug study. Furthermore, we develop a method for systematic validation of drug-induced changes in the cardiac excitation-contraction coupling.

To evaluate the suitability of hiPSC-CMs for predictive safety pharmacology, we quantified changes in contractility, voltage and Ca<sup>2+</sup> handling simultaneously in 2D monolayers with our in-house developed Triple Transient Measurement (TTM) system (van Meer et al., 2019). Furthermore, we developed an hypothesis-based statistical algorithm that identifies mechanisms of action. Subsequently, we evaluated a set of drugs with known positive, negative or neutral inotropic effects.

Using the TTM system we were able to identify 78% of the expected drug-induced effects accurately in our *in vitro* model.



Furthermore, we were able to pick up over 80% of the mechanistic changes in excitation-contraction coupling. While we will continue to strive for a 100% accuracy, these results indicate hiPSC-CM based microphysiological systems can be a serious alternative to certain animal models currently used. Rewarded with the Hugo van Poelgeest Price, this work forms the basis of ongoing research identifying whether certain animal models can be replaced in cardiotoxicity testing.

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### Mechanistic and integrative strategies for identifying thyroid-active chemicals impacting environmental and human health

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To support the EU strategy on endocrine disruptors, and JRC and OECD work on the methodology for their assessment, eight projects were funded by the EU Horizon 2020 Research and Innovation Programme and form together the EURION cluster. Within EURION, three projects focus on novel improved approaches for the identification of thyroid hormone (TH) disruptors.

The ERGO project aims to identify and develop TH disruption-related biomarkers and endpoints for linkage of effects between vertebrate classes. The strategy is based on Adverse Outcome Pathway (AOP) network covering various modes of TH disruption. An *in vitro* bioassay battery is set up to address identified cross-species priority molecular initiating/key events and to support *in vivo* studies. The models for studying the prioritized endpoints include human/mammalian cell lines from thyroid, liver and neural stem cells.

The ATHENA project develops new testing strategies for the identification of chemicals that adversely affect fetal brain devel-

opment by changes in TH availability. The *in vitro* studies focus on: 1) high-throughput screening of large compound libraries for inhibition of deiodinases, dehalogenases and cell membrane TH transporters, yielding training sets for QSAR development; 2) assays for disruption of TH membrane transporters in physiological barriers in placenta, brain, and choroid plexus; 3) 3D models for disruption of neural cell fate using mouse neurospheres and iPSC derived human brain organoids.

The SCREENED project develops *in vitro* assays based on rodent and human thyroid cells organized in 3D constructs to mimic the structure and function of the native thyroid gland. These include 3D organoid based on stem cell derived thyrocytes (SCDT) and on decellularized thyroid stromal matrix repopulated with SCDT, and bioprinted constructs based on SCDT able to mimic the spatial and geometrical features of a native thyroid gland. These constructs are hosted in modular microbioreactors with sensing technology and controlled culture conditions.

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### Retrospective evaluation of animal and non-animal modelling: Efficacy-related examples informing organisational practices and driving improvements

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Research into clinical phase, efficacy-related attrition in the pharmaceuticals sector has been partly linked in publications and analyst reports to an erosion of confidence in animal studies as a predictive modelling platform (Harrison, 2016). GSK is working to optimise the translational relevance of preclinical modelling strategies and improve future delivery of successful medicines to patients. Specifically, the GSK Animal Research Strategy team has supported this by leading efforts to leverage retrospective knowledge, from animal and non-animal models and by sharing learnings with stakeholders to continuously improve modelling practices in the organisation.

Efficacy-focused case examples will be shown from small molecules and biologics that reached clinical phases (predominantly phase II) and have been evaluated retrospectively via After Action Reviews (AARs) for preclinical-clinical concordance. Through integration of biology/pathobiology and “pillars of pharmacology” evidence (focusing on animal and non-animal models) and by relating this to clinical trial designs and outcomes, these reviews highlighted several themes for continuous improvement. These included biological and technical aspects, increasing alignment preclinically to clinical intent, and differ-



ences between endpoints measured and hypotheses articulated. This work also highlighted opportunities to integrate information across disciplines and solved perceived challenges in re-using historical information and appreciating past decisions.

The approach taken in the AARs resulted in a robust process to access and evaluate relevant content from projects retrospectively.

The experiences from the AAR subset are being applied to inform and improve current practice, shape ongoing strategy to continually improve animal research, advance non-animal considerations, story-tell potential challenges to discovery leaders and to shape organisational practices around decision-making and data capture.

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## A case study combining read-across and NAM, the example of propylparaben in systemic toxicity, from A to Z

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The Long Range Science Strategy (LRSS) is Cosmetics Europe (CE) research program on new generation risk assessments (NGRA) based on new approach methodologies (NAMs). It includes a series of case studies, and one of them is on parabens. We use a tiered workflow, coming from the Seurat-1 program and published by the OECD. The goal is to perform a safety assessment of parabens with long or branched lateral chain based on existing information from the parabens that have short lateral chain and for which toxicokinetics and toxicodynamics information are available. At Tier 0 we collected

information on use scenario and chemical features. Parabens are Cramer Class I, but the exposure exceeds by far the threshold of toxicological concern (TTC) value. Read across shows robust studies available with a need to prioritize developmental and reproductive toxicities. In Tier 1 we focused on determining the bioavailability of the parabens, and thus the systemic concentration. We used *in vitro* experiments (e.g., plasma protein binding, hepatic clearance in human hepatocytes) to parametrize a physiologically based biokinetic (PBBK) model. These data have the potential to be used for a conclusion based internal TTC (iTTC), and a dedicated LRSS project is under development. We moved to Tier 2, where the focus is on specific targeted testing, to also explore modes of action and toxicodynamic (e.g., docking studies, ToxCast<sup>®</sup> data, endocrine receptor assays, transcriptomics). The conclusion on the safety, here, is based on read across using both chemical and biological similarities.

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## Semantic modelling of adverse outcome pathways and the implementation in reproducible workflows

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The purpose of Adverse Outcome Pathways is to organize mechanistic knowledge on toxicological processes upon exposure to a stressor leading to an Adverse Outcome in a series of Key Events (KEs). It aims to facilitate the replacement, reduction, refinement (3Rs) of animal testing in risk assessments (Ankley et al., 2010; Burden et al., 2015). Qualitative descriptions of AOPs are generally stored in the public AOP-Wiki. However, its content remains relatively isolated and is only manually queryable or through downloading the full dataset.

We tackled this AOP-Wiki limitation by FAIRifying into the AOP-Wiki Resource Description Framework (RDF), including semantic annotations and persistent identifiers for chemicals and proteins. The RDF allows flexible queries to extract information through a SPARQL endpoint, e.g., from coding environments, such as Jupyter notebooks. As a demonstration, we developed a notebook that adds information to an AOP of interest and finds and extracts supporting experimental data from ToxCast and TG-GATES based on the molecular targets and stressor chemicals, and identify the affected molecular pathways underlying KEs using WikiPathways (Slenter et al., 2018).



Overall, the AOP-Wiki RDF improves the accessibility and interoperability of the database, providing additional ways of querying the data and enabling the implementation in automated workflows. By focusing on increasing the machine-readability of AOPs and using consistent vocabularies and ontologies, AOPs become an increasingly useful tool for integrating toxicological knowledge and data, thereby increasing the effectiveness of the concept and addressing the 3Rs in toxicological studies.

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## Dam transportation, fetal suffering and legal objections – Why fetal bovine serum should be a thing of the past

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Fetal bovine serum (FBS) is a supplement widely used in cell and tissue culture to enhance cell growth and division, despite its many scientific disadvantages being widely discussed in the scientific community (van der Valk et al., 2018). Over and above this, ethical and legal considerations should play a more substantial role in the discussion on the use of FBS. This is because FBS is derived from the blood of bovine fetuses after their removal from the slaughtered dam. Fetal blood is harvested by cardiac puncture. This is usually performed without stunning or anesthesia of the fetus, resulting in massive ethical and animal welfare concerns, as potential pain and suffering cannot be excluded (van der Valk et al., 2004). But ethical considerations should not only include the fetuses but start as early as with the dams, as trans-

portation of pregnant cows can cause distress and suffering and can trigger (premature) birth and even abort, especially in the late stages of gestation.

Moreover, blood harvesting for FBS production lacks binding regulations, resulting in a legal grey area and therefore opens the door for mistreatment or even fraud to the detriment of animals, scientists and patients.

However, alternatives to FBS do exist and further use and development of ethical acceptable substitutes should be promoted. In our presentation we argue that instead of justifying FBS collection as a necessary evil and continuing to use a product that is questionable for a variety of reasons, the way forward should be a substantial change that starts from the way we treat farm animals and spans the replacement of FBS by alternatives that are more humane and scientifically sound.

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## Key learnings from *in vitro* vapor and direct liquid exposure studies for acute respiratory toxicity

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The 3M Strategic Toxicology Laboratory is working to incorporate *in vitro* screening Air Liquid Interface (ALI) tools to assess the acute respiratory toxicity potential for new chemicals. Studies have examined multiple aspects of the experimental designs, including different ALI cell culture systems, dynamic vapor and liquid phase exposure methods, viability measurements and post-exposure periods. The goal is to better understand the critical parameters of the cell systems and exposure methods to enable the development of a consistent screening model, while gaining insight into the dosimetry. Acrolein has been used as a model respiratory toxicant to help evaluate the various parameters. With acrolein, the two different ALI models, A549 and Epi-Airway™, showed similar responses, with cell viability (based on MTT) decreasing as a function of acrolein exposure concentration, both during dynamic vapor exposure and direct liquid exposures. Post-exposure viability determination has shown to be a critical parameter for evaluation, as viabilities generally decreased from immediate post-exposure to the 20/24-hour post ex-



posure time-point. Sequential liquid exposures produced EC50 values well aligned with dynamic vapor exposures, compared to single liquid exposures. Quantification of the vapor concentrations during exposure has been important to understand the dosimetry relationship between liquid and vapor phase exposures. Direct mathematical conversion from a liquid ( $\mu\text{g/mL}$ ) into vapor (ppm in air) concentration results in thousands-fold higher exposure estimates compared to vapor concentrations that elicit the same effects on cellular viability; however, calculation of exposure on a mass per tissue surface area basis resulted in meaningful comparison between these two modes of exposure. These data are valuable to understanding and effectively communicating the results of different *in vitro* ALI respiratory toxicity models using vapor and liquid phase toxicant exposure.

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## SAToRI-BTR: Developing preliminary guidance on evaluating quality and human relevance of *in vitro* studies in brain tumour research

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**Background:** Considerable interest exists in the potential of *in vitro* studies to address questions related to clinical use of drugs and the pathobiology of tumours (NC3Rs, 2020). Agreement is, however, required on how to assess quality, quantity and human relevance of such studies alongside adequate reporting (Hartung et al., 2019). The SAToRI-BTR (Systematic Approach To Review of *In vitro* methods in Brain Tumour Research) project focuses on seeking consensus as to how brain tumour studies using *in vitro* methods should be evaluated.

**Objectives:** To identify criteria for evaluating quality and human relevance of *in vitro* brain tumour studies; to assess the acceptability of such criteria to senior scientists in the field.

**Methods:** Potential criteria for evaluation were identified through: an online survey of brain tumour researchers; interviews with scientists, clinicians, regulators, and journal editors; analysis of relevant reports, documents and published studies. A preliminary set of criteria for quality appraisal was compiled through content analysis. In stage two, the criteria were reviewed by an expert panel (Delphi process).

**Results:** Methods for and quality of review of *in vitro* studies were found to be highly variable with a need for improved reporting standards. 129 preliminary criteria were identified; duplicate and highly context-specific items were removed, result-

ing in 48 criteria for review by a panel of senior researchers from 9 countries. 38 criteria reached consensus, resulting in a provisional checklist for appraisal of *in vitro* studies in brain tumour research.

**Conclusion:** Through a systematic process of collating criteria and subjecting these to expert review, SAToRI-BTR has resulted in preliminary guidance for appraisal of *in vitro* brain tumour studies. Further development of this guidance, including investigating strategies for adaptation and dissemination across different sub-fields of brain tumour research, as well as the wider *in vitro* field, is planned.

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## The OECD IATA Case Studies Project – Five years of shared experience in the integration of new methods in a regulatory context

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Integrated Approaches to Testing and Assessment (IATA) are pragmatic, science-based approaches for chemical hazard characterization that rely on an integrated analysis of existing information coupled with the generation of new information using testing strategies. IATA follow an iterative approach to answer a defined question in a specific regulatory context, considering the acceptable level of uncertainty associated with the decision context. IATA range from more flexible, non-formalized judgment-based approaches to more structured, prescriptive, rule-based approaches and can include a combination of methods from one or many methodological approaches.

The OECD IATA Case Studies Project provides a forum for countries to explore the use of novel methodologies in IATA within a regulatory context. There is a scientific exchange on how novel methods are applied to assess the hazard of chemicals and establish common approaches for the use of these methods.

The project was launched in 2015 to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. This project reviews case studies submitted from member countries and stakeholders every year. The review results are discussed in a



project meeting. More than 20 case studies have been reviewed and the lessons and learnings summarized in consideration documents (see: <http://www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm>).

Most IATA approaches discussed have involved the use of new approach methods (NAMs) to support read-across within chemical categories or between analogues. Increasingly, the strategies are also driven by knowledge of adverse outcome pathways (AOPs) or knowledge of the mode of action. In addition, the use of information on metabolism is being applied to inform grouping and read-across. The case studies draw on multiple methods from *in silico*, *in vitro* and existing *in vivo* data to build weight of evidence approaches to inform chemical assessment.

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## Modeling blood-brain barrier permeation in the autologous stem cell-derived Chip4

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Over the last years microphysiological systems have been increasingly accepted by academia and industry as a valuable tool in drug development to test substances for their safety and efficacy. Current systems still face the problems of heterogeneous tissue sources, hindering the development of patient specific chip systems. To overcome these limitations, we recently developed the autologous TissUse Chip4 combining miniaturized human intestine, liver, brain and kidney equivalents derived from one single human induced pluripotent stem cell (hiPSC) line (Ramme et al., 2019).

Understanding the ability to pass the blood-brain barrier (BBB) is crucial for assessing safety and efficacy in the development of neurological-active compounds. Therefore, we have enhanced our neuronal model (Koenig et al., 2018) by introducing blood-brain barrier specific endothelial cells (BBB-ECs).

Here, we present results from the adaption of the new neurovascular model to the Chip4. Furthermore, we show results of first tests with propranolol and atenolol and their metabolism and permeation across the model of the BBB. To produce enough neuronal models, we use a large-scale neuronal induction protocol in a fully defined and controlled DASbox<sup>®</sup> bioreactor system. The neuronal spheroids are then combined in a commercial Transwell<sup>®</sup> model with state-of-the-art hiPSC-derived BBB-ECs (Appelt-Menzel et al., 2017; Lippmann et al., 2015), to separate the neuronal compartment from the rest of the system. The BBB-ECs form a tight monolayer on the Transwell membranes

as shown by trans-endothelial electrical resistance and low sodium fluorescein permeation and maintain their phenotype in the Chip4. Furthermore, metabolism and permeation characteristics of propranolol and atenolol were comparable to the *in vivo* situation underlining the high physiological relevance of the system.

In future, we are planning to use the Chip4 to generate relevant PBPK data of test substances including intestinal uptake, metabolism, blood-brain barrier permeation/transport and renal absorption.

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## Remove ATT, TABST & LABST. How far away are we to global harmonization for those safety tests?

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Many institutions and organization have been working independently or jointly to remove those obsolete safety testing from the production and batch release testing for human (ATT) and veterinary vaccines (TABST, LABST). Many regulatory agencies and international organizations have successfully introduced waivers or have deleted them (Viviani et al; Lei et al). How far away are we from the global elimination of those tests? What is the role of international organizations in promoting the change towards animal-free safety testing? Humane Society International is presenting its work under the Animal Free Safety Assessment Collaboration (<http://www.afsacollaboration.org>) to engage industry and regulators in countries like Russia, India, China, South Korea, Brazil to promote this important change that



reduces the burden on animals, the final costs of the vaccines, and accelerates their release time to the market.

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## Integrating innovative methods to predict a regulatory hazard endpoint: A regulatory perspective

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The United States Environmental Protection Agency's (U.S. EPA) Office of Pesticide Programs (OPP) regulates the use of all pesticide chemicals. To evaluate potential risks to humans, the OPP evaluates exposures from multiple routes as part of the human health risk assessment. Whole animal studies are typically required and/or used to evaluate inhalation exposures; however, regulatory statutes provide the EPA with the flexibility to modify the actual studies required on an individual chemical basis. Therefore, the Agency may use data from alternative methods and strategies to satisfy data requirements. The EPA's strategy to reduce animal testing relies heavily on the development and implementation of new approach methodologies (NAMs). NAMs have been adopted as a broadly descriptive reference to any non-animal technology, methodology, approach, or combination thereof that can be used to provide information on chemical hazard and risk assessment. This presentation will discuss the process taken thus far by the U.S. EPA, including challenges and the Agency's needs, to implement an alternative approach to refine inhalation risk assessment for a contact irritant.

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## SEARCHBreast: A virtual bioresource to facilitate the sharing of surplus animal materials derived from breast cancer studies

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The contributions that animal models have made to oncology research are well recognised and documented. Following this has been a strong desire to observe best practice in the use of animals in cancer research and the Workman guidelines (Workman et al., 2010) have impacted on researchers aiming to address the 3Rs (Replacement, Refinement, and Reduction). However, there is significant surplus material generated from most animal studies and this is often archived in-house, stored indefinitely and frequently never re-visited, hence representing a considerable untapped resource. We used this opportunity to develop a virtual bioresource called SEARCHBreast (Sharing Experimental Animal Resources, Coordinating Holdings – Breast) which makes what is frequently a hidden resource of leftover material derived from animal studies in breast cancer more visible and accessible to scientists. I will outline the development of SEARCHBreast and describe some of the challenges faced in its adoption by the breast cancer research community.

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**Presentation:** Oral

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## Fish cell lines of rainbow trout as alternatives to fish in environmental risk assessment: Where we stand and where we need to go

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Millions of fish are used every year in the safety testing of chemicals and water samples. Three OECD certified tests are most commonly employed: fish acute toxicity test (OECD 203), the



fish early life stage test (OECD 210) and a test to determine chemical bioconcentration factors (OECD 305). In order to start reducing or even replacing these tests, we have developed experimental strategies that evolve around permanent cell lines of rainbow trout (*Oncorhynchus mykiss*), one of the species suggested in the above-mentioned OECD guidelines.

Chemicals are assumed to be taken up mainly via the gill or the intestine. The liver, on the other hand, is considered as a major site for chemical biotransformation. Thus, we focus our research on three cell lines derived from rainbow trout gill (RTgill-W1), intestine (RTgutGC) and liver (RTL-W1). Exposure of cells to chemicals is combined with quantification of chemical exposure and with mechanistic computational models as appropriate. As a result, we have devised strategies to predict:

- (i) fish acute toxicity using the RTgill-W1 cell line. The so-called RTgill-W1 cell line assay has undergone validation (Natsch et al., 2018; Tanneberger et al., 2013) and round-robin testing (Fischer et al., 2019); it has been adopted by ISO (ISO standard 21115) and by OECD (April 2021) as the first *in vitro* ecotoxicology test guideline;
- (ii) bioconcentration factors based on chemical biotransformation rate constants derived with the gill, the gut and the liver cell line (Stadnicka-Michalak et al., 2018);
- (iii) chemical impact on fish growth based on RTgill-W1 cell population growth over the course of several days in the absence or presence of chemical (Stadnicka-Michalak et al., 2015).

These approaches will be introduced and the journey to international standardization and regulatory acceptance discussed using the RTgill-W1 cell line assay as example.

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**Presentation:** Oral

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## The integration of *in vitro* chemical transplacental passage into a generic PBK model for pregnancy

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With the increasing application of cell culture models as primary tools for predicting chemical safety, the quantitative extrapolation of the effective dose from *in vitro* to *in vivo* (QIVIVE) has become increasingly important. For developmental toxicity this requires scaling the *in vitro* observed concentration effect levels to *in vivo* fetal exposure, by integration of *in vivo* kinetics, including information on transplacental transfer. This transport of substances across the placenta barrier, has been studied here with the use of the BeWo cell system, an *in vitro* model mimicking *in vivo* passage of a chemical from maternal blood across a one-cell barrier into the embryo. Six model compounds with known embryotoxic potential have been applied. The *in vitro* BeWo assay results were thereafter incorporated as generic QIVIVE of transplacental transport of xenobiotics in Physiologically Based Kinetic (PBK) modelling. For this purpose, an existing generic PBK model was extended specifically for the rat pregnancy. This included the adaptation of the model to account for maternal body changes during pregnancy, as well as the addition of a specific fetoplacental sub-compartment. The obtained PBK-predictions were compared with *in vitro* developmental toxicity data of the respective chemicals, as taken from the open literature. The BeWo results illustrated different transport profiles of the chemicals across the BeWo monolayer, allocating the substances into two distinct groups: the “quickly-transported” and the “slowly-transported”. Exposure PBK-simulations during gestation demonstrated satisfactory kinetic predictions, at least for some of the compounds, when compared to experimentally measured maternal blood and fetal concentrations. Comparisons of PBK-predicted concentration-response curves with *in vitro* concentration-response curves, advocate the C<sub>max</sub> or average exposure as the best dose metric for developmental toxicity, depending each time on the chemical. Overall, the *in vitro* to *in vivo* comparisons suggest a promising future for the application of such approaches in chemical risk assessment.

**Presentation:** Oral

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## Human iPSC cell-based models for predictive toxicology

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To improve alternatives to animal testing, new approach methodologies has been emerged to provide information in terms of chemical hazard and risk assessment. To date, many strategies, such as human induced pluripotent stem cell (iPSC)-based models, organ-on-a-chip (also called micro physiological system), mini organs that more closely mimic native tissue function has shown great potential for various applications, such as safety assessment. Based on two-dimensional (2D) differentiation protocols from iPSCs to mimic features of tissues *in vitro*, *in vitro* safety studies using iPSC-cardiomyocytes have demonstrated their ability to inform on drug-induced cardiotoxicities (Kanda et al., 2018; Blinova et al., 2018). In addition, iPSC-neurons are expected to provide mechanistic data toward developmental neurotoxicity testing. Recently, increasing attention has been paid to iPSC-derived organoids or 3D culture system that are mini-organ models to recapitulate human tissue function (Yamada et al., 2019). However, these 3D organoid models are various and have not been validated well. Good cell culture practice should be considered for the organoids (Pamies et al., 2017). Thus, effective utilization of these advances requires an understanding of their advantages and limitations for practical application. Here I would like to provide an overview of how to generate human organoids *in vitro* and discuss the exciting challenges for human iPSC-based models and future perspectives toward safety assessment.

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**Presentation:** Oral

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## Identification of appropriate methods for severity assessment in a widely used mouse model of acute colitis

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**Introduction:** According to the EU Directive 2010/63, recognizing and minimizing pain, suffering, and harm of laboratory animals is a legal requirement and essential for both scientific and ethical reasons. To detect signs of diminished well-being, it is crucial to choose sensitive parameters which allow the required classification into mild, moderate, or severe experimental severity. We compared and assessed the respective merits of different behavioral and physiological methods for severity assessment in laboratory mice in a model of chemically induced, acute colitis in an effort to refine this model.

**Methods:** 12-week-old female C57BL/6J mice were exposed to 0.0 %, 1.5 %, or 2.5 % dextran sulfate sodium (DSS) via drinking water for five consecutive days. Over the course of disease, we compared species-specific burrowing behavior and a composite pain score with physiological parameters like body weight, clinical scoring, corticosterone levels, and fecal occult blood.

**Results:** The acute colitis had an observable impact on burrowing behavior, the composite pain score, and other physiological parameters. With the lower dose of 1.5 % DSS, the burrowing tests and the composite pain score were superior in detecting disease severity, whereas body weight remained stable. No impact on corticosterone was detectable and fecal occult blood did not correlate with colitis severity.

**Conclusion:** Physiological parameters and clinical scoring should always be monitored in severity assessment. Nevertheless, behavioral tests will provide a substantial added value to the refinement. Changes in burrowing behavior reflected pain that was not evident in clinical scoring. Additionally, the composite pain score detected impairment of animals earlier in the course of colitis than clinical scoring or body weight.

**Presentation:** Oral



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## The monocyte activation test (MAT) for medical devices: An alternative test method for the detection of pyrogen- and material-induced immune activation

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The safety of medical devices is the top priority for manufacturers, but the diversity of materials and applications complicates regulations and necessary tests. Usually, the length and nature of the contact to the human body define the risks and limits. Contaminating pathogens on medical devices, e.g., bacteria or yeasts, and their components (e.g., endotoxin, lipoteichoic acid, zymosan, flagellin) (Stoppelkamp et al., 2016) have the ability to induce fever due to their pyrogenicity. In some cases, the materials themselves can trigger a pyrogenic/inflammatory response (Trunk, 2019). In the ISO 10993-11 guideline Appendix G (specifying tests for systemic toxicity) pyrogenicity is divided into either endotoxin-induced or material-induced, where material-induced pyrogenicity is summarized as being derived from “non-endotoxin related factors” (DIN EN ISO 10993-11:2017, Biological evaluation of medical devices). These factors are to be tested by the rabbit pyrogen test (RPT), while endotoxins are to be tested by an endotoxin test. The listed non-endotoxin related factors are endogenous pyrogens, prostaglandins, exotoxins, neurotransmitters, inductors (e.g., polyadenylic acid), some metals and substances such as morphine, dinitrophenol, and naphthylamine. No reference is made to non-endotoxin pyrogens (e.g., zymosan, etc.) and abiotic substances. For the latter, in particular, the material-induced pyrogenicity should be taken into account. Our laboratory has applied the MAT on diverse medical devices and showed that an inflammatory response from pathogen-derived endotoxins and non-endotoxins can be distinguished from material-induced pyrogenicity. Moreover, the intended use of a particular medical device should be taken into account when judging the pyrogenic risk. Cotton-based abdominal swabs, for example, are commonly used during surgery and their excellent adsorptive and hemostyptic properties are valued. In cardiac surgery using the heart-lung machine, however, activated blood could re-enter circulation and start an inflammatory cascade. In this session, data of different medical device categories tested by the MAT will be shown and the potential use of the method to replace RPT for non-endotoxin pyrogens discussed.

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**Presentation:** Oral

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## Organoid-based expansion of airway epithelial cells from clinical samples with low cell numbers for modelling effects of cigarette smoke exposure

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The effect of exposure to airborne toxicants such as those present in cigarette smoke (CS) can be studied in airway epithelial cells (AEC) cultured at the physiological air-liquid interface (ALI), but establishment of such cultures requires substantial cell numbers that cannot always be obtained from patients. Recent studies show that 3D organoid cultures can be initiated from samples with limited AEC numbers, such as bronchoalveolar lavage (BAL; Sachs et al., 2019). Aim of the present study was to compare expansion of AEC using 3D organoid culture to conventional 2D plastic-based expansion for establishment of ALI cultures, and study whether such cultures can be used for exposure to CS. AEC were obtained from tracheal aspirates (TA) from preterm newborns, and from BAL or bronchial tissue (BT) from adults. TA and BAL cells were 3D-expanded, whereas cells from BT were expanded in 3D and 2D. Following expansion, AEC were cultured at ALI to induce differentiation. The impact of cell origin and 2D or 3D expansion was assessed with respect to (i) cellular composition; (ii) response to CS exposure; (iii) effect of Notch inhibition or IL-13 stimulation on cellular differentiation. Well-differentiated ALI cultures were established from all samples and cellular compositions were comparable. All 3D-expanded cultures showed a similar oxidative stress and unfolded protein response following CS exposure, but different from the 2D-expanded cultures. Additionally, TA- and BAL-derived



cultures were less sensitive to modulation of differentiation by DAPT or IL-13 than BT-derived cultures. We conclude that organoid-based expansion of clinical samples with low epithelial cell numbers, such as TA from preterm newborns, is a valid method to establish ALI cultures, and that the method of expansion affects the response to CS.

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**Presentation:** Oral

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### Optimization of an *in vitro* placental transfer assay for screening purposes

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Embryotoxicity is an essential toxicological endpoint of chemicals and drugs. Alternative methods to animal testing are being developed for developmental toxicity and applied already for screening purposes. Nevertheless, without transfer of an active substance via the placenta, potential direct effects on the embryo can be neglected. Therefore, considering the kinetic parameter “placental transfer” is necessary. The *in vitro* placental transfer model can be used to determine the placental transfer rate by determining the apparent permeability coefficient (Papp value) of a substance.

Using a trophoblastic cell line (BeWo b30) on a transwell system, a cell barrier is formed separating the apical (representing maternal side) from the basolateral (representing fetal side) compartment. However, varied protocols exist with insufficient characterization. In this study methodology is optimized (weekend-free time schedule) and characterized to enable screening processes.

Characterization parameters included are trans-epithelial electrical resistance (TEER), fluorescein transfer (paracellular control), histology, immunohistochemistry of cell tight junctions and transfer of permeability controls amoxicillin (low) and antipyrine (high). The optimization alters the frequency of medium change. Stable cell layer integrity was observed on day 6. The TEER increased from day 3 to day 6 reaching a value of  $\sim 40 \Omega \cdot \text{cm}^2$ . This increasing was followed by a decreased of up

to 80% on the Fluorescein transfer, confirming the barrier integrity. The Papp values of permeability controls amoxicillin ( $10.4 \pm 2.3 \text{ cm/s}$ ) and antipyrine ( $48.3 \pm 6.4 \text{ cm/s}$ ) were in the literature range for both methodologies. Determining the relative amoxicillin Papp to the reference compound antipyrine ( $22 \pm 3\%$ ) facilitates the comparison among substances in different run experiments or laboratories and guarantee reproducibility and the setup of historical control data.

The established protocol may be combined with other *in vitro* or *in silico* strategies as an animal-free approach to assess developmental toxicity.

**Presentation:** Oral

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### Transferability challenges with modern technology and how to overcome them – A case study of the GARD assays from a test method developer’s perspective

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Predictive toxicology is currently going through paradigm shifts, both relating to the movement from animal-based assays towards *in vitro*, *in chemico* and *in silico* methods, and the increased focus on mechanistic understanding of investigated toxicological endpoints. This shift is accelerated by innovative predictive methods, oftentimes associated with substantial investments in research and development. While sufficient levels of transparency, reliability, transferability and test method availability are required for regulatory acceptance, an opportunity for test method developers to get a return on investment should be secured.

The case of skin sensitization constitutes an example of promising implementation of alternative methods in a regulatory framework. To this end, the Genomic Allergen Rapid Detection (GARD) platform is a next-generation *in vitro* testing strategy framework for assessment and characterization of chemical sensitizers. The GARD platform integrates state-of-the-art technological components, including omics-based evaluation of transcriptional patterns of endpoint-specific genomic biomarker signatures, machine learning-assisted classification-models and a streamlined analysis pipeline facilitated by cloud-based software.

Currently, both the GARDskin and the GARDpotency assays are the subject of review for regulatory acceptance. While the submitted and published data clearly demonstrates added values that positively contribute to existing and proposed strategies for hazard and risk assessment and characterization, the reliance on advanced technological components have also posed specific challenges for method transferability and regulatory acceptance.



In this session, we share our view of such hurdles and how they can be overcome. The presentation will be based on real case experiences acquired throughout the validation processes. The specific challenges discussed relates to topics such as intellectual property right protection, GLP-compliance of cloud-based applications, prediction model transparency and investments associated with instrumental hardware. In summary, we illustrate the importance of continuous dialogue and collaboration between stakeholders in order to ultimately benefit both the innovation sector and the society at large.

**Presentation:** Oral

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## RHE-LC, reconstructed epidermis including Langerhans cells, a useful tool for skin immunity study and evaluation

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Langerhans cells are epidermal antigen-presenting cells. They are key players in a wide range of immune-mediated skin responses and skin disorders. Availability of reconstructed human epidermis containing Langerhans cells opens new possibilities for research and development in dermatology and cosmetics.

SkinEthic™ RHE-LC (EPISKIN SA) model is reconstructed on a polycarbonate filter membrane by seeding human primary keratinocytes with CD34+ derived cells and cultivated for 17 days. Characterization of the model is done by histology and immunostaining (CD207) and Transmission Electron Microscopy (Birbeck granules). Functional responses of the resident Langerhans cells are assessed by RT-qPCR studies (CCR7 and CD86) after topical or systemic exposure of the tissues to chemical sensitizers (PPD, DNCB, Isoeugenol).

Immunolabelling of the SkinEthic™ RHE-LC tissues with CD207 antibodies show regular repartition of Langerhans cells in the reconstructed epidermis. 24 h hours after exposure to sensitizers, Langerhans cells activation is shown by the upregulation of CCR7 and CD86 expressions.

These results show that SkinEthic™ RHE-LC is a functional model of human skin able to mimic some mechanisms of human exposure to skin sensitizers. The SkinEthic™ RHE-LC model is therefore expected to be a useful tool for skin immune response studies.

**Presentation:** Oral

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## Re-homing rodents: Opportunities and challenges

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The EU Directive 2010/63/EU (EU, 2010), which is implemented by all member states, stipulates that animals used or intended to be used in procedures may be re-homed, provided that the health state of the animal allows it, that there is no danger to public health, animal health or the environment, and that the well-being of the animal is safeguarded. The Australian Code for the Care and Use of Animals for Scientific Purposes (Australia, 2013) goes beyond that, stating that opportunities to re-home animals should be considered wherever possible. As far as we know, no other legislation or guidelines on laboratory animals include a paragraph on the re-homing of laboratory animals. Nevertheless, good re-homing schemes contribute to a culture of care that many research institutes are attempting to cultivate.

Existing re-homing schemes have largely focused on species such as dogs, cats, and non-human primates, with good results. But what about rodents? Should we promote that rodents are included in institutional re-homing schemes? Can we safely re-home rats and mice, and if so, how?

During 2019 and 2020, the Utrecht Animal Welfare Body has started re-homing rodents in close cooperation with several animal welfare organizations. In this presentation we will discuss the criteria necessary for a successful re-homing scheme for rodents. We will offer insight in our strategies and partnerships, and share the views and experiences from animal caretakers, adopting homes and intermediary organizations, including the media.

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**Presentation:** Oral



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## Integrating toxicokinetics and toxicodynamics for decision-making in an NGRA context: 2 Cosmetics-Europe case studies

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In the last 5 years, initiatives on decision-making supported by New Approach Methodologies (NAMs) have made progress and delivered several pragmatic tools. In addition to the capability building effort, other challenges related to increasing confidence and fostering the implementation of these approaches globally are being addressed. The Cosmetics Europe Long Range Science Strategy (LRSS) is an initiative that aims to develop human-relevant safety assessment approaches for cosmetic ingredients. Case studies are the backbone of the science strategy. They offer the opportunity to proof check the validity of the approaches being developed, while highlighting strengths and limitations of the NAMs used and gaps which warrant further effort. For systemic toxicity, different types of safety assessment are addressed within the LRSS, including the threshold of toxicological concern (TTC), development of an internal TTC (iTTC), next-generation read-across and *ab initio* approaches.

We have conducted case studies based on caffeine and propylparaben, which illustrate the use of NAM-based toxicodynamic information combined with toxicokinetics for systemic toxicity assessment. The safety assessment is exposure-led, hypothesis-driven and builds on a Next Generation Risk Assessment (NGRA) workflow. One feature of the NGRA is to derive margins of internal exposure using bioactivity data from NAMs in combination with exposure. It is hoped that the learnings from these case studies will be leveraged to elaborate guidance documents on applying NGRA.

**Presentation:** Oral

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## The biotransformation and bioaccumulation of ionizable organic compounds in rainbow trout cell lines

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The assessment of chemicals for their bioaccumulative potential requires *in vivo* testing with fish. These tests are resource intense, costly, time consuming and of high ethical concern due to the sacrifice of animals. Therefore, alternative *in vitro* models are being sought to replace these tests.

Recently, it has been demonstrated that *in vitro* bioassays with rainbow trout (*Oncorhynchus mykiss*) cell lines from liver (RTL-W1), gill (RTgill-W1) and gut (RTgutGC) can be used to determine biotransformation rate constants and, by means of *in vitro-to-in vivo* extrapolation, predict bioconcentration of a neutral organic compound (benzo(a)pyrene) in rainbow trout (Stadnicka-Michalak et al., 2018a). However, the usability of these cell lines to assess the biotransformation and bioaccumulation of ionizable organic compounds (IOCs) has not yet been investigated. IOCs comprise a large proportion of the chemicals in commerce (Franco et al., 2010) and are ubiquitously detected in the environment and biota (Schlüsener et al., 2015; Munz et al., 2018). Therefore, we have started to explore how well fish cell lines represent the uptake of IOCs into fish. Based on the availability of high-quality *in vivo* data and the substance's charge state at physiological pH, three cationic and four anionic substances were selected. Among the selected IOCs are pesticides, pharmaceuticals and surfactants. In a first step, non-toxic chemical concentrations were determined (Stadnicka-Michalak et al., 2018b) and chemical analytical procedures established such that chemical starting concentrations can safely be tested at concentrations at least 20x above quantification limits. Cell exposure is then performed over 48 h, during which cells and exposure medium are sampled to derive chemical specific uptake and elimination rates. These rates are used in a toxicokinetic model to derive bioconcentration factors and compared to *in vivo* bioconcentration factors from literature. The conceptual approach and first kinetic rates and bioconcentration factors will be presented.

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**Presentation:** Oral

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## Animal-free testing of cell-based medicinal products

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Cell-based medicinal products (CBMPs) consist of viable cells of human origin. Different animal species display different cell surface markers, activation factors, expression pattern of specific genes, and possess distinct immune system components making testing for toxicity challenging.

A project was launched under auspices of the Ministry of Agriculture, Nature, and Food Quality of the Netherlands, with the objective to study the possibility whether CBMPs could be developed safely for human use without animal testing.

By analyzing CHMP Scientific Advice reports written for 84 CBMPs in 2013 to 2019, our research revealed that approximately 70% of human cell products were tested in animals for toxicity. Only 5-12% of total products were tested with animal-free methods. No animal toxicity studies were performed with dendritic cell-based products. The performed toxicity studies were general toxicity (55%), immunogenicity (29%), safety pharmacology (19%), and a very low number of reproductive toxicity studies (2%). Animal models were roughly divided into rodents (mice or rats), large animals (pigs, sheep, goats, horses, and dogs), and non-human primates.

When analyzing the safety package of products without *in vivo* toxicity studies, we found that the informativeness of the safety package predominantly relied on already available clinical experience and subsequently on *in vitro* safety studies.

The observed adverse effects in the *in vivo* safety studies that were most commonly discussed were pulmonary thrombosis, effects caused by cell entrapment due to high doses, immunological effects, and effects that were difficult to interpret.

Thus, clinical data played the most crucial role in terms of product safety assessment. We envisage that a gradual shift toward animal-free safety testing of CBMPs will be the future trend in the development of such products.

**Presentation:** Oral

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## Rehoming rodents – Perspective and experience of an animal welfare organisation

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Animals that are notably useful for humans/human health like laboratory animals and as a (negative) consequence, are denied a species-appropriate life, are particularly gladly supported by animal welfarists. Adopters are therefore usually happy when rehoming them and willing to give a lot in return.

Generally, high demands are placed on rehoming laboratory animals (e.g., specific expertise, suitable housing conditions, well-planned procedures). Rehoming concepts must be species-specific including different requirements for the donor, intermediary organizations/persons as well as new owners. Aspects relating to animal health, prior castration, transport equipment, species-specific knowhow, rehoming experience, animal-friendly husbandry etc. must be taken into account. New animal owners must be carefully selected, while ensuring high standards of housing conditions, species-knowledge, sufficient time as well as species-appropriate husbandry conditions. In addition, it must be ensured when rehoming through animal welfare organizations that acceptance for control services (before and after the animals are handed over) as well as signing protection treaties is given.

Successful rehoming projects require sensitivity, good communication and cooperation from all parties involved. Last but not least, financial considerations and agreements are also required. Currently rehoming projects are financed privately mainly by animal welfare organizations. The adopters also make substantial contributions. Sometimes also researchers contribute with private funds.

From an animal welfare point of view, rehoming-projects must ensure, in the sense of a comprehensive culture of care concept, the whereabouts of laboratory animals after experimentation are well thought out and rehoming is taken into account whenever possible. Rehoming funding must be given space, ideally already proactively together with the submission of the application for the animal experiment project. Private funding should be seen



very most as initial funding and be replaced by an adequate research budget.

In this talk I will present you our concept and experiences of rehoming laboratory rats, mice and rabbits.

**Presentation:** Oral

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## Continuous training of animal welfare body members – An education program for oversight on welfare and care of laboratory animals

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Directive 2010/63/EU assumes an animal welfare body that must follow the development and outcome of projects at establishment level, foster a climate of care and provide tools for the practical application and timely implementation of recent technical and scientific developments in relation to the principles of replacement, reduction and refinement, the 3Rs, in order to enhance the life-time experience of the animals. Therefore, according to art 26.2 the animal-welfare body must include the person or persons responsible for overseeing the welfare and care of the animals and, in the case of a user, a scientific member.

Since 1977 the Netherlands has an act on laboratory animal experiments. In the decree on animals used in animal experiments (1985) training requirements for an animal welfare officer were described such as: Master's degree on (veterinary) medicine, or biomedical science, PhD and/or experienced with laboratory animal science experiments, supplemented with a special postdoctoral laboratory animal science course (26 weeks).

The revised Dutch Act on Animals used for scientific purposes (2014) combines the Directive to the already present requirements for animal welfare body with art13f3a: a person responsible for overseeing the welfare and care of the animals. The qualifications for this art13f3a person however have not been described. In practice, the Netherlands brings the responsibility as described in art 26.2 together with the implementation of over-

sight on quality on scientific research, i.e., competent personnel, implementation of the 3Rs and experimental design for every experiment.

A group of people already in charge for the responsibilities as mentioned has taken the initiative to make an education program for art13f3a officers to stimulate and harmonize a high standard for culture of care and animal experimentation in all establishments in the Netherlands. The need for substantial qualification to oversee animal welfare and quality of animal research will be discussed.

**Presentation:** Oral

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## Development of a human bone-on-a-chip to model intramembranous ossification in basic science and toxicology

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Bone is surprisingly dynamic and harbors a variety of cell types. Unlike other tissues, it is constantly renewed by a coordinated interaction of bone-forming (osteoblasts) and bone-resorbing (osteoclasts) cells in a process called remodeling. It is thus challenging to recreate bone and its developmental steps *in vitro*. Hitherto, most of the current models focus on aspects relevant only within a certain biological context and do not consider all key features of bone like mechanical stress, hypoxia, the composition of the extracellular matrix and its different cell populations (Scheinpflug et al., 2018). Consequently, animal models are still the gold standard to explore bone biology and pathology, although it becomes more and more evident that species-specific differences in physiology hamper the translation of results obtained from animal models to humans (Knight, 2007).

In our project, we aim to establish a 3D human *in vitro* model to simulate bone development in a more physiologic manner. Therefore, we combine a 3D aggregate, generated from primary osteoblasts, with an in-house designed micro-physiological system, able to regulate oxygen saturation and mechanical load, to create a “bone-on-a-chip”. We established two protocols for aggregate generation. One approach is based on bio-assembly strategies, starting with osteoblast aggregates, while the other approach uses 3D printing for organoid generation. Our data demonstrate a donor-dependent variation of mineralized matrix components for the different bio-assembled samples. In printed aggregates, we observed an elevated alkaline phosphatase activity as well as an extracellular increase in inorganic phosphate



in static culture when compared with samples within the micro-physiological system. In addition to alterations in the cell's morphology as shown by cytoskeleton staining, this could indicate the induction of osteocytogenesis within the micro-physiological system.

In the long term, such a "bone-on-a-chip" may provide a meaningful alternative to reduce and replace animal testing in basic research and toxicology.

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**Presentation:** Oral

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## NC3Rs resources to improve the reproducibility of *in vivo* and *in vitro* experiments

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Many factors influence the reproducibility of preclinical experiments, with issues around suboptimal experimental design and incomplete reporting estimated to account for half of irreproducible research (Freedman et al., 2015). Researchers can have limited knowledge and understanding of experimental design and statistics and may not appreciate the importance of rigorous methodology. This has an impact on the way they design, conduct and report experiments, and it also affects the way they assess manuscripts and grant proposals in their capacity as journal editors, reviewers and funding panel members. The NC3Rs has developed a suite of tools to support researchers to improve. This includes the recently revised ARRIVE guidelines (Percie du Sert et al., 2020b) and accompanying Explanation and Elaboration document (Percie du Sert et al., 2020a), and the online Experimental Design Assistant which provides bespoke feedback on individual experimental plans (Percie du Sert et al., 2017). These resources provide extensive guidance on how to design, conduct and analyse animal experiments, and what crucial information to report in scientific publications. Additional resources are currently being developed to improve the design and reporting of *in vitro* research.

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**Presentation:** Oral

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## DORA declaration, open science and its impact on the assessment of (animal)research

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The San Francisco Declaration on Research Assessment (DORA) is a worldwide initiative published in 2012. It aims to focus on a broader set of assessment criteria for research and researchers, rather than purely focusing on bibliometric indicators like citation numbers or the H-index. By doing this, the goal is to ensure that hiring, promotion and funding decisions are based on the qualities of research that are most desirable, namely insight, impact, reliability and re-usability.

In 2019, also the Dutch Royal Academy of Science (KNAW), the Dutch Research Council (NWO) and ZonMw1 signed the Declaration. By endorsing the DORA principles and the movement towards Open Science, ZonMw commits itself to work on adapting current assessment procedures. For a selection of programs, the DORA principles were implemented, asking applicants to describe their academic profile in narrative form. The narrative addresses collaborations, educational activities, research focus, vision, and good research practices instead of their usual publication output alone. Changes in research assessment should also impact the way we assess (animal) research. For example, specific assessment criteria for animal-based research can be whether or not preregistration of animal studies is performed, whether Publication and Prepare guidelines (e.g., ARRIVE and PREPARE) were followed or whether all results are published (neutral/negative) by the researcher (see also abstract #951). Another potential criterion is whether systematic review of animal studies has been conducted to support the chosen model and hypothesis of grant applications. Some potential assessment criteria are currently being piloted within a few funding programs.



As it is work in progress, the definitive list of assessment criteria is not yet determined. You are therefore cordially invited to discuss with us what criteria you think should play a role to improve insight, impact, reliability and re-usability of animal-based research and animal free innovations.

**Presentation:** Oral

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## Achieving reproducibility through responsible animal research

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Improving reproducibility in preclinical studies will require many individuals to take action. But how is this achievable when we all work in different environments and have varying levels of knowledge, experience, habits, pressures and support?

One practical tool that can help is a checklist. They break complex tasks down into an ordered sequence of steps that transforms understanding and makes consistent results achievable. Checklists clarify what is required to: help us be organised; motivate us to take action and complete tasks; improve efficiency and reduce stress by supporting us to make less mistakes; improve self-confidence and support individuals to implement good practice. They are a valuable tool that puts everyone on the same level, without judgement or presumptions.

What checklists cannot do is: make individuals read or implement them, provide the answers or replace the thought processes that individuals need to go through when planning, conducting, analysing or communicating their research. These aspects are influenced by the local research culture and the individuals within it.

In this talk I will share with you my personal checklist for responsible animal research with links to freely available tools (MERIDIAN, 2020), resources (PREPARE, 2018) and guidance (UKRIO, 2019; UKRN, 2020) to help incorporate it into our daily research practices. I will also share what I believe to be key points to consider in relation to how good training and support programmes can influence local research cultures (Insight blogs, 2020).

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**Presentation:** Oral

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## Harmonisation of the three Rs in biologicals

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The European Partnership for Alternative Approaches to Animal Testing (EPAA) project team on Global Harmonisation of 3Rs in Biologicals is working towards harmonization of testing requirements for vaccines and other biological products in different geographies. The team is working since 2015 to encourage the deletion of general safety tests (GST, e.g., abnormal toxicity tests (ATT) in mice or guinea pigs, target animal batch safety tests (TABST)) for vaccines from legal requirements and guidance documents, such as pharmacopoeia monographs, WHO recommendations, and OIE guidelines. Advanced in-process controls, validation of the manufacturing process and release testing complying with international standards are also part of modern vaccine development and render the general safety test in animals obsolete. The presentation will summarize progress in this area since the WC10 in 2017. Key achievements were the complete removal of the ATT from European Pharmacopoeia monographs, the WHO ECBS recommendation to discontinue inclusion of the GST in all future WHO Recommendations, guidelines and manuals for biological products, reference to VICH guidelines 50 & 55 being introduced in the OIE manual and the deletion of the ATT or allowance for waivers when consistent production is proven, e.g., India, Brazil, Argentina, South Africa. Ideas for tackling the remaining harmonization challenges in this area will be discussed.

**Presentation:** Oral



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## Advancing three Rs under a European Parliament pilot project

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The European Parliament provided funding to the European Commission under a Pilot Project to promote the use of alternatives to animal testing in the EU through information sharing and education activities. The project aims to promote existing alternatives, facilitate development and validation of new alternatives, foster exchanges of information, knowledge and best practices and to provide tools for education and training to facilitate the application of the Three R's principle – to Replace, Reduce and Refine the use of animals used for scientific purposes – in line with Directive 2010/63/EU. Today's users and future scientists are the targets of this project. The Pilot Project consists of three separate, identifiable pillars:

1. To develop six open access (on-line) eLearning modules of which two will focus on alternatives (“How to search for non-animal alternatives” and “Development of non-animal alternatives with a view to their use in regulatory context”); the other four modules will focus on the implementation of the Three Rs covering “project design” (levels I and II), “project evaluation” and “application of severity assessment framework”. These modules will help providing much needed consistency across the EU on key elements that are crucial for the correct application of the legislation.
2. To support the Education and Training Platform for Laboratory Animal Science (ETPLAS), which links Member State authorities, course providers and course accreditors, as a one-stop-shop for information on training and assessment tools for those tasked with evaluation of Learning Outcomes and competence assessment. ETPLAS will also host the six eLearning modules.
3. To develop guidance for decision-makers in educational organizations to facilitate the incorporation of the Three Rs into their curricula at three levels of education, i.e., high schools, universities and professional education.

**Presentation:** Oral

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## Reimagining preclinical studies through digital transformation; leveraging computer vision, machine learning, mixed reality & informatics platforms to maximize data quality and clinical relevance of preclinical studies

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As new technologies emerge, it is imperative to keep abreast of them so that we can leverage them for animal wellbeing and scientific impact of animal studies. Computer vision and machine learning allow for continuous and noninvasive animal monitoring for changes in behavior, physiology and environment. These monitoring technologies provide an opportunity to optimize pre-clinical toxicology and efficacy studies by collecting physiologically and translationally relevant data, assessing disease states and safety more objectively and with quantitative measures. Tele-clinical assessment allows animal technicians to connect with veterinarians and scientists in real-time and expedite decisions. Integration of these technologies into informatics platform makes animal data accessible, retrievable and learnable, and increase efficiencies of daily processes. Engaging with these technologies enhances clinical assessments and training and results in real-time actionable insight, faster decisions, reduced biocontamination and noncompliance risks, increases harmonization and clinical relevance.

**Presentation:** Oral



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## Establishing scientific confidence in a cell-free method to predict acute inhalation toxicity

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Acute inhalation toxicity testing for regulatory purposes relies on the use of rodents according to the OECD test guidelines 403, 433 and 436. Apart from the ethical concerns of exposing animals to toxic chemicals, *in vivo* testing is expensive and time-consuming. In addition, the use of death or evident clinical signs of toxicity gives no insight about the mechanism of toxicity of the tested chemicals. Alternative methods are much needed. 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 inhalation toxicity will be discussed. The method addresses the first key event of the newly proposed adverse outcome pathway AOP 302, starting from the molecular initiating event interaction of a substance with lung surfactant and leading to the adverse outcome of acute inhalation toxicity. Indeed, lung surfactant is the first barrier that inhaled substances encounter once at the alveolar airspaces. Its main function is to regulate the surface tension at the respiratory air-liquid surface to avoid alveolar collapse *in vivo* and, thereby, acute inhalation toxicity. Insights in the mechanism of toxicity of three chemicals towards the lung surfactant complex at the molecular level will be shown using a combination of imaging techniques and biophysical assays. 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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## Acute inhalation toxicity after inhalation of ZnO nanoparticles: Lung surfactant function inhibition *in vitro* correlates with reduced tidal volume in mice

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ZnO nanoparticles (NPs) exposure, by inhalation of consumer products or during industrial processes, can induce acute inhalation toxicity. The toxicological mechanisms underlying the acute effects on the lungs have long focused on the phagolysosomal dissolution of ZnO NPs in macrophages followed by the release of free Zn<sup>2+</sup> ions. We propose an alternative mechanism based on the direct interaction of ZnO NPs with the lung surfactant (LS) layer covering the inside of the alveoli. This hypothesis was tested by 1. investigating the effect of both ZnO NPs and Zn<sup>2+</sup> ions on the function of LS *in vitro* using the constrained drop surfactometer, and 2. examining the role of lung macrophages in the acute toxicity of inhaled ZnO NPs. LS function was inhibited in a concentration-dependent manner during exposure to ZnO NPs *in vitro*, whereas exposure to Zn<sup>2+</sup> ions did not show any effect on the function. Our results suggest that the ZnO NPs themselves, rather than the free zinc ions, are the causative agent for acute inhalation toxicity. *In vivo*, mice were treated with Clodrosome<sup>®</sup>, a drug that depletes alveolar macrophages, or Encapsome<sup>®</sup>, the empty carrier of the drug. After macrophage depletion, the mice were exposed to an aerosol of ZnO NPs in whole body plethysmographs and the breathing patterns were recorded continuously. Both groups of mice treated with Clodrosome<sup>®</sup> or Encapsome<sup>®</sup> developed shallow breathing (reduced tidal volume) shortly after the onset of exposure to ZnO NPs. Thus the mechanism of acute toxicity of inhaled ZnO NPs was macrophage-independent.

**Presentation:** Oral



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## Optimal duration of safety studies with monoclonal antibodies

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In order to evaluate the safety and efficacy of new drugs, it is often necessary to conduct animal studies according to international guidance. Stakeholders are increasingly aware that this must be done in line with 3Rs principles. Re-evaluation of regulatory guidance (a 4<sup>th</sup> R) can further provide opportunities to restrict the use of animals in safety and efficacy studies to those which provide meaningful information that is relevant to humans. This EPAA-supported project aims to analyze whether long term toxicity studies (up to 6 months duration) with monoclonal antibodies identify new relevant safety findings. A preliminary evaluation of marketed products suggests that long term studies may not always be needed. In collaboration with EPAA industry partners, NC3Rs and MEB, a survey was developed to identify whether long term safety studies of non-marketed molecules, as well as marketed products, result in novel relevant safety findings. The consortium consists of 15 companies from Europe and USA with commitment to submit anonymized study data for compounds with short- and long-term studies in relevant toxicology species. Based on these data, the consortium aims to:

1. Establish criteria on the basis of which decisions can be made on the need and duration of non-clinical safety studies for monoclonal antibodies, based on approved and non-approved drug development programs
2. Establish regulatory consensus based on scientific facts that these criteria are acceptable as a justification to deviate from the guidelines in future marketing authorization applications
3. Initiate discussions to document these criteria in regulatory guidance documents

**Presentation:** Oral

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## In vitro irritation testing of medical devices: Validation and acceptance

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Assessment of dermal irritation is an essential component of the safety evaluation of medical devices. In 2016-2017, an international round robin validation study was conducted to determine if reconstructed human epidermis (RhE) assays could be an acceptable replacement for the rabbit skin irritation test required by ISO 10993-10. Two RhE models were evaluated: EpiDerm™ (MatTek, Inc.) in 18 laboratories and SkinEthic™ RHE (EpiSkin, SA) in eight laboratories. Four irritant polymers and three non-irritant controls were obtained or developed and certified prior to use. Blinded polymer samples were extracted with sesame oil and saline per ISO 10993-12. The apical surfaces of tissues were dosed with 100 µL extract aliquots. Positive and negative solvent controls were included. Tissues were kept in humidified incubators at 37°C with 5% CO<sub>2</sub>. Incubation times were 18 hours (EpiDerm™) and 24 hours (SkinEthic™ RHE). After incubation and rinsing with PBS, cell viability was determined by the colorimetric MTT reduction method. Cell viability reduction greater than 50% was indicative of skin irritation. Both the EpiDerm™ and SkinEthic™ RHE tissues were able to correctly identify virtually all of the irritant polymer samples either in the saline, sesame oil or both solvent extracts. These results indicate that RhE tissue models can detect the presence of strong skin irritants at low levels in dilute medical device polymer extracts, and therefore are suitable replacements for the rabbit skin irritation test. This work was published in an August 2018 open access special issue of Toxicology In Vitro. In addition, the new ISO 10933-23 standard on irritation testing of medical devices was based on our results. This global standard, which will be published in late 2020, states that RhE *in vitro* assays are the preferred method for irritation testing of medical devices.

**Presentation:** Oral

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## Education and training to fully implement refinement methods in practice

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Retrospective reviews of the implementation of refinement methods in practice revealed that the knowledge we have gained from refinement research is not fully applied in practice and thus, laboratory animals may endure unnecessary suffering (Herrmann and Flecknell, 2018a,b, 2019). Experimental refinements, including those related to anesthesia and analgesia protocols, less-inhumane endpoints and killing methods, were reviewed in over 500 basic and applied animal research proposals. Furthermore, a literature review was conducted to assemble the latest best practice-approaches in regard to housing and care. In all areas of refinement reviewed, inadequacies in the application of available approaches that could help to reduce unnecessary pain, distress and suffering of animals used in science were detected. One potential explanation that was identified in the review was that researchers might not be sufficiently aware of existing refinement methods. This situation is understandable considering a recent international survey, conducted by the European Commission's Joint Research Centre, which found that very few courses could be identified at university level that are specifically teaching the 3Rs (Herrmann, 2019). To help remedy this situation, an 8-week module-based course for university students and early career scientists is being established at Johns Hopkins University to teach about best practice-approaches in refinement and to foster a culture of care for laboratory animals. Several modules cover housing and experimental refinement; others address what might be termed refinements of planning, including identifying the most appropriate research model, analyzing data properly, and reporting results comprehensively. Also discussed are the consequences of poor refinement method use for the animals' welfare and for the validity of collected data. The lectures are complemented by interviews with experts in laboratory science to explore reasons for the currently low refinement implementation rates and to find possible approaches to improve the situation.

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**Presentation:** Oral

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## Skin sensitization testing strategy for Japan

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Recently, various *in vitro* skin sensitization tests have been listed in the OECD Test Guidelines. However, skin sensitization process cannot be covered by a single *in vitro* test due to its complexity. Therefore, the Guidance document based on integrated approaches to testing and assessment (IATA) has been published by OECD, and the Guideline is being developed. Here, Kao's ITS and STS models and Shiseido's artificial neural network (ANN) model are introduced as examples of IATA case studies developed in Japan. The sequential testing strategy (STS) is a straightforward decision tree based on DPRA and h-CLAT (addressing Key Events 1 and 3 of the AOP). The approach predicts three LLNA potency classes (strong, weak, and non-sensitizing). The integrated testing strategy (ITS) is a scoring-based decision tree that uses data from DPRA and h-CLAT and Derek Nexus, and covers Key Events 1 and 3. These two approaches were selected as candidates of defined approaches for the OECD Guideline. The ANN model is a non-linear statistical model that combines multiple *in vitro* and *in silico* parameters covering Key Events 1-3. The model predicts the LLNA EC3 value. In 2018, the Ministry of Health, Labor and Welfare, Japan published a Guidance on an evaluation system that combines multiple *in vitro* tests for the evaluation of safety for cosmetics and quasi-drugs. Only when all three tests are negative, skin sensitization could be judged as negative. This defined approach is called bottom-up 3 out of 3 model and has predictivity with very few false negatives but potentially many false positives. In addition, two defined approaches were included in the Guidelines for Agricultural Chemical Registration Application issued in 2019. Current situation in Asia (e.g., South Korea, China and ASEAN) will be introduced, too.

**Presentation:** Oral



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## Alternatives to animal testing in vaccine manufacturing and release

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On average, Virbac Australia sells 32 million doses of livestock vaccines per year. Those vaccine doses in turn protect about 29 million animals (cattle and sheep) per year. Every batch of vaccine manufactured and sold by Virbac Australia undergoes stringent tests to ensure their safety, efficacy and quality. Most of these tests, especially tests for product release (potency testing), are performed according to international regulatory guidelines, such as the British or European Pharmacopoeia or the USDA's guidelines as laid down in Code of Federal Regulations 9. These guidelines unfortunately prescribe the use of significant numbers of laboratory animals. In line with the 3R principles to replace, reduce and refine animal use, Virbac Australia is committed to reducing (and eventually eliminating entirely) the number of animals used in the manufacture and release of vaccines. Virbac has an ongoing program to develop, validate, register and implement *in vitro* assays to replace our current animal-based tests. This presentation focuses on the first phase of Virbac's *in vitro* program, which involves the development, validation and implementation of immunoassays as replacements for product potency tests requiring LD50-type assays. The *in vivo* potency tests to be replaced by immunoassays are: i) mice serum neutralization tests, which evaluates the antibody response of vaccinated rabbits in mice by assessing the dilution of rabbit serum which can protect mice from lethal amounts of toxin; and ii) guinea pig challenge, where vaccinated guinea pigs are challenged with virulent cultures of *Clostridium chauvoei*. The latter is a prescribed product batch release potency test for Blackleg vaccine. We believe Virbac is the first Australian manufacturer to locally register and use a non-LD50-type assay to release Blackleg vaccine. Here we describe the development, validation and discussions with the Australian Pesticides and Veterinary Medicines Authority (APVMA) leading to registration.

**Presentation:** Oral

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## Establishment of a comparative *in vitro* assay for developmental toxicity evaluation by assessing fetal anemia using embryonic erythroid cells derived from human and rat pluripotent stem cells

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To assess developmental toxicity of chemicals is of significant importance. However, the relevance of animal testing for the evaluation of the effects of chemicals in humans is limited because of the species differences between humans and animals, including biosynthetic pathways. For example, some N-phenylimide herbicides are reported to cause developmental toxicity in rats but not in rabbits by suppressing heme production in embryonic erythroid cells. In this study, we established a comparative *in vitro* assay for developmental toxicity evaluation by assessing fetal anemia using embryonic erythroid cells differentiated from human induced pluripotent stem cells (hiPSCs) and rat embryonic stem cells (rESCs). Erythroid cells obtained from hiPSCs and rESCs by our newly refined differentiation method showed erythroid characteristics, e.g., heme production and/or glycophorin A expression, and had the embryonic feature of expressing a high level of  $\epsilon$ -globin. These embryonic erythroid cells derived from hiPSCs and rESCs were treated with flumioxazin, a widely used N-phenylimide herbicide that causes rat-specific developmental toxicity, and dihydroartemisinin (DHA), an anti-malarial drug that causes depletion of embryonic erythroid cells in various species tested. As a result, a dose-related reduction in heme synthesis occurred in rat erythroid cells treated with both flumioxazin and DHA. This result was consistent with the reported *in vivo* data obtained from rats. In contrast, flumioxazin had no effect on either heme synthesis or cell proliferation in human cells, although DHA caused a reduction in cell number and heme content. These results indicate a possible qualitative difference between human and rat erythroid cells in their response to flumioxazin and the improbability of anemia leading to developmental toxicity in human embryos. This study demonstrated the usefulness of this pluripotent stem cell-based comparative assay system for future toxicological and mechanistic studies of developmental toxicity.

**Presentation:** Oral



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## 21<sup>st</sup>-century toxicology and regulatory testing: An update from Japan

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The Japanese Center for the Validation of Alternative Methods (JaCVAM) has a dual purpose: to promote the Replacement, Reduction, and Refinement in animal experiments for the evaluation of chemical substance safety in Japan, and to establish guidelines for new alternative experimental methods through international collaboration. Many areas of toxicity testing, such as genotoxicity, pyrogenicity, phototoxicity, skin sensitization, and eye or skin irritation/corrosion, have ongoing test method validations being coordinated by JaCVAM. These validation studies include international experts on every management team, and are in various stages of development, where some have recently led to regulatory adoption via the Organization for Economic Cooperation and Development (OECD) Test Guidelines and/or government recommendations.

For the systemic toxicological endpoints of repeated dose toxicity, immunotoxicity and developmental toxicity, new test methods are expected to be developed in the future worldwide. I believe Japan will make a significant contribution to these developments in the Japanese projects such as the OECD projects, the AI-SHIPS project – a METI contract research project to develop a state-of-the-art, AI-based chemical safety prediction system using big data, the Japan Agency for Medical Research and Development (AMED) Regenerative medicine projects and AMED – the Microphysiological Systems (MPS) project.

**Presentation:** Oral

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## The transition to animal-free innovation: The Utrecht approach

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The Dutch government has established a Transition Programme for Innovation without the use of animals (TPI). The TPI programme is bringing people and organizations together in order to accelerate innovation towards better science with less animals.

Utrecht University, the University Medical Centre Utrecht and the University of Applied Sciences Utrecht have decided to actively embrace the challenge and have recently founded a joint TPI Utrecht working group, with ambassadors who represent the

different research fields. The aim of TPI Utrecht is to effectively support and further boost the transition.

TPI Utrecht has the ambition to create a safe environment for all parties involved, that all strive for scientific excellence. We recognize that diversity makes us stronger, and therefore seek out inclusion of differences. We insist on a culture of respect and recognize that words and actions matter. Communication is a central element in all our activities.

TPI Utrecht will have a dense agenda of inspirational sessions driven by our ambassadors of innovation; connection with the several research groups will create out-of-the box ideas and new opportunities for grant application geared to achieve better human and animal health. We strongly believe in innovation of education: we will work on new technology and curricula.

In our presentation, we will share our strategy, our short term and long terms plans, and our accomplished and ongoing activities. We will talk about our struggles and seek for feedback and collaboration.

**Presentation:** Oral

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## A European Commission funded project to develop learning outcomes and assessment tools to facilitate harmonization of LAS education and training in Europe

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In the EU, Education and Training are the responsibility of the individual Member States (MSs). Standards may differ between MSs, affecting the free movement of personnel. Course certificates obtained in one MS may not be accepted by the authorities of another MS. In 2018, the European Commission awarded a grant to ETPLAS, the Education and Training Platform for Laboratory Animal Science, to develop Learning outcomes and assessment tools for LAS education and training in line with the EU Education and Training Framework guidance. Five working packages (WPs) were created with representatives from stakeholder groups such as course providers and accreditation bodies. Their respective objectives are to develop guidance for producing assessment criteria of Learning Outcomes (LOs), to set-up a database of assessment criteria for core modules and function A specific modules, to establish a question database for theoretic



cal core and function A specific modules, to establish a database of assessment of common practical tasks (DOPS) for Function A persons, and to establish the required IT infrastructure on the ETPLAS platform. The deliverables of WP 1 through 4 will be made available through this new ETPLAS web-portal. Now, one year into the project, a progress report will be presented.

**Presentation:** Oral

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## Applying the 3Rs within regulatory toxicology studies in drug development

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The UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (<http://www.nc3rs.org.uk>) convenes international consortia to discuss opportunities to apply the 3Rs within toxicology programmes. Expert working groups of industry scientists, academics and regulators take an evidence-based approach to recommend efficiencies in study designs and promote best practices to minimise animal use and refine procedures that enhance animal welfare. Two case studies will be presented.

A recent data-sharing project involved 36 pharmaceutical companies and regulatory bodies from the UK, Europe and USA (Prior et al., 2020). Data on 172 compounds from 18 companies were analysed to investigate opportunities to use one, rather than two species for toxicity testing. The number of species and the target organ toxicities identified per species in different duration studies were determined. Results indicate that reduction to one species for longer-term toxicity studies (as per ICH S6(R1)) could be applicable for a wider range of biologics, and small molecules / other modalities where two species toxicity testing is currently recommended.

We continue to monitor the impacts of our first cross-company data-sharing activity, which questioned the value of stand-alone acute toxicity studies during pharmaceutical development (Robinson et al., 2008). These studies were historically required via two routes of administration to support the registration of any new medicine, to identify doses that caused major adverse effects and the minimum dose causing lethality. However, the group showed that more useful information was provided from other nonclinical studies (e.g., short-term maximum tolerated dose studies) and the acute tests were removed from ICH M3 guidelines in 2009. As a result, inclusion of acute oral toxicity data in UK clinical trial applications submitted to the MHRA has fallen from 86% of submissions in 2007 to almost zero in 2019.

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**Presentation:** Oral

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## Current status of the OECD project on integrated approaches to testing and assessment for acute fish toxicity

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The acute fish toxicity test (OECD Test Guideline 203), which uses 96 h lethality as the endpoint, is required in various regulatory frameworks and is one of the most frequently used aquatic toxicity tests. This represents a very significant animal welfare concern. Moreover, while knowledge and concerns about the scientific limitations of individual fish tests in terms of reproducibility and relevance to environmental protection are growing, more scientifically advanced methods are being developed and improving toxicity assessment. Therefore, Integrated Approaches to Testing and Assessment (IATAs) for acute fish toxicity are of high environmental, scientific and social interest. IATAs structure and guide the combination of existing information, computational tools, mechanistic information from the molecular and cellular levels (e.g., using fish cells), information from species of lower



trophic levels such as Daphnia and algae, and refinement methods using fish at life stages when they are less susceptible to pain and suffering than in adulthood (e.g., fish embryos). Through the OECD Test Guidelines Programme, Austria and the International Council on Animal Protection in OECD Programmes are working together with leading experts and the OECD VMG-Eco, to develop such IATAs. The related OECD project aims to enrich the Threshold Approach for Acute Aquatic Toxicity Testing (OECD Guidance Document 126) with new toxicological tools. The outcome of the research project “Strengthening Weight of evidence for FET data to replace acute Fish Toxicity (SWiFT)” funded by the CEFIC Long-range Research Initiative (LRI ECO 51) will feed into the OECD project. In this presentation, progress towards developing IATAs will be discussed.

*Disclaimer: The views, conclusions and recommendations presented are those of the authors and do not necessarily represent the policies or positions of the organizations to which they are affiliated.*

**Presentation:** Oral

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## Improved product characterization using non-animal methods: Development of an immunoassay for diphtheria and tetanus vaccines

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Batch release testing for human and veterinary vaccines relies heavily on methods that involve animals – particularly for potency testing. Despite recent advances in the development of methods that refine or reduce animal use, there have been fewer developments that have resulted in complete replacement of animals used for vaccine batch release. Our objective, as part of the VAC2VAC project, is to develop an immunoassay for assessment of antigen content and quality in diphtheria (D) and tetanus (T) vaccines as an indicator of potency and to substitute for existing animal potency tests.

Relevant monoclonal antibodies (mAb) were selected for use as critical reagents in the assay based on their ability to bind native and/or detoxified antigen, adsorbed antigen and antigen that was altered following exposure to elevated temperature. Neutralization tests were also used to select antibodies that target a relevant functional epitope on the antigen. Affinity measurement and epitope competition studies were performed using Biacore to identify pairs of high affinity antibodies that have been used to develop a sandwich ELISA for D and T.

The mAb ELISA can detect antigen in a wide range of vaccines for human (D, T) and veterinary (T) use. This includes antigen detection in the final lot in the presence of non-aluminium and aluminium based adjuvants. The assay is quantitative and can identify changes in antigen content for a vaccine that has been deliberately formulated to contain a graded series of antigen concentrations. The assay can detect antigenic changes following exposure of non-adjuvanted toxoids and final lot vaccines to elevated temperature, suggesting that the ELISA may be stability indicating.

Suitably validated immunoassays, such as the ones described here may be able to substitute for existing *in vivo* potency assays as part of a consistency approach.

**Presentation:** Oral

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## Reducing animal use in pharma by integrating electroencephalogram, behavior & cardio-hemodynamic readouts in freely moving rodents

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Although alternatives for animal research are rapidly evolving, in many conditions, animals cannot be fully replaced therefore continued efforts should be taken to Refine and Reduce the total numbers of animals.

In drug development, one of the top priorities is to select safe new drug candidates. One of the major problems, is the appearance of drug-induced seizure/convulsions. The full complexity of this liability is not easily predicted from 2-D cell models. Consequently, rats are traditionally used for the examination of central nervous system (CNS) mediated behavioral, neuromuscular, autonomic and sensorimotor effects. Although liability for drug induced tremors/convulsions are explored in these observational studies they are not suited for detection of seizures that do not develop convulsions. For these conditions electroencephalogram (EEG) assessments are needed (Authier et al., 2016). However, these are only applied when neurological concerns are identified. A second limitation of the traditional studies lies within the interobserver variability and limited observation attention span. These could be improved with evolving computerized behavioral assessments.

Apart from CNS effects, hemodynamic liabilities are a great drug development concern. Chronic blood pressure/heart rate increases can increase morbidity & mortality in the clinic and should therefore be avoided (Picard et al., 2011).

To address these welfare concerns while at the same time reducing and refining the animal studies, we aimed to combine



CNS and cardio-hemodynamic endpoints in one experimental *in vivo* study approach. Accordingly, we applied a radio telemetry platform for CNS and cardiovascular endpoints together with automated behavioral and temperature assessment. This method allows continue measurements of EEG and cardio-hemodynamics in combination with behavioral video-monitoring, while rats are in pairs in their home-cage, without experimenter interference. The advantages of this model include an improvement of the animal welfare with a decrease in stress levels, together with a reduction on the animals used.

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**Presentation:** Oral

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## Towards an animal-free human health assessment: What are the regulatory needs?

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Societal concern for animal welfare and scientific concerns about the predictive power of animal models are driving forces for the development of animal-free approaches for the safety testing of chemicals. Despite the many interesting innovations, a paradigm shift towards an assessment of human health risks fully based on animal-free approaches is not foreseen within the next decade. To accelerate the use of animal-free innovations in the EU, it has multiple advantages to work bottom-up towards a new safety assessment paradigm and to aim their development at better meeting the current regulatory needs. The question then is what these needs are and what regulatory questions need to be answered? In relation to chemical safety assessment, management and communication, a large number of different questions can be posed that may require the development of different tools and assessment strategies (Bos et al., 2020). For instance, what basic information is needed within the context of the following areas of chemical safety assessment in the EU: 1) classification, labeling and packaging, 2) the derivation of health-based guidance values and product limits, 3) risk assessments of exposure situations of concern and 4) addressing specific topics of societal

concern. Agreements on the level of detail and uncertainty, robustness, predictive value, reproducibility and validation are a prerequisite to develop tools that can be trusted and that will be legally binding. A challenging aspect is to go beyond the present regulatory requirements and to develop animal-free innovations that can address exposure scenarios that vary in exposure route, intensity (dose, concentration), duration and frequency, including short-term and long-term high exposures, following e.g., accidental release. To develop innovations that are fit-for-purpose, a multi-stakeholder collaboration is needed already in the development phase of animal-free innovations, where regulators can inform on the regulatory needs and the criteria for acceptance.

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**Presentation:** Oral

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## EU expert working group proposals for common guidance on the creation and breeding of genetically altered animals (GAAs)

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In 2017, around 30% of the 9.38 million animals used in the EU were genetically altered, with the proportion higher for mice 36%, and zebra fish 64%. Also, a further 1.3 million animals were used in the creation and breeding of Genetically Altered Animals (GAA), and 6.1 million GAA reported as surplus in 2017 (EC, 2020; [https://ec.europa.eu/environment/chemicals/lab\\_animals/other\\_reports\\_en.htm](https://ec.europa.eu/environment/chemicals/lab_animals/other_reports_en.htm))

The welfare and care of GAA animals are therefore important issues to consider in the context of animals bred and used for scientific procedures.

In 2012, following a report from an Expert Working Group (EWG), the National Contact Points of the Member States of the EU endorsed a document providing guidance on the use of GAAs under Directive 2010/63/EU ([https://ec.europa.eu/environment/chemicals/lab\\_animals/pdf/corrigendum.pdf](https://ec.europa.eu/environment/chemicals/lab_animals/pdf/corrigendum.pdf)).

As GAA use increased, and new technologies in the creation of GAAs evolved, the European Commission convened, in 2018, a further EWG to update the 2012 document. Furthermore, the requirements for the classification of different GAA lines for the purposes of project authorisation and reporting of GAAs had been identified as causing difficulties during the recent review of the Directive.



The EWG focussed on the development of welfare assessment schemes for the most commonly used GA species, and the methods by which such welfare information could be shared with scientists using the GA animals, to promote informed husbandry and care practices, and to allow addressing specific needs as a result of GA.

The EWG also identified and made recommendations regarding the key Three R elements which should be considered during the creation and breeding of GAAs.

The final elements considered by the EWG related to the administrative requirements relating to the authorisation and reporting of GAAs.

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#### Presentation: Oral

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## Distance learning resources to support training of animal welfare body members

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Members of animal welfare bodies (AWB) have varied backgrounds and experience and hence have diverse training needs. They often have limited time available to undertake training, and within an institution the number of individuals that need training is usually small. This can present a challenge when trying to deliver face-to-face training of individuals. We strongly advocate providing such face-to-face learning opportunities, for example through training workshops, but distance learning solutions can complement these activities.

We have developed two e-learning modules, with the support of the European Commission, which will be of particular value in both the introductory and ongoing training of AWB members.

The first module which was designed primarily for project evaluators (EU25) has many aspects relevant to AWB members. The second is a new EC module (EU26) which covers the assessment of severity in detail. This module has been developed for those with responsibilities for conducting or having oversight of research projects that use living animals. These two modules will be available for use, with open access, from early 2021.

We have also trialed the delivery of training to AWB members using webinars, with recordings available for download and review after the sessions.

This seminar will provide examples of the materials and discuss some of the practical and technological concerns associated with the delivery of training using online methods.

#### Presentation: Oral

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## Technology is moving GSK towards the substitution of animal-testing

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GSK identifies, develops and manufactures innovative medicines, vaccines and consumer healthcare products. GSK's animal studies are conducted with high standards of humane care and treatment. These studies represent a small but vital part of our procedures to develop future products and perform the mandatory release of vaccines. GSK's moral responsibility has resulted in a company-wide strategy to reduce animal use by developing non-animal technologies (NAT) to test the safety and efficacy of new and existing vaccines. This 3R commitment is aligned with the active engagement in collaborative studies to sponsor 3R and to develop and validate NAT as alternatives to the use of animals.

Continuous engagement with external partners to assess technical solutions ensures a proactive reduction. Together with an annual systematic review on the animal use drives a strong effort on replacing animal testing in GSK's global vaccines strategy.

To guarantee transparency and compliance GSK actively communicates 3R efforts with regulatory authorities. In our engagement we aim to co-build NAT by aligning industry and authority requirements to ensure the safety of the patient and to design robust methods for Quality release.

GSK's internal 3R program, has reduced the use of animals by 30% since 2016 and is expected to reach 75% by 2025 in Vaccines Quality Control. This is achieved for *in vivo* potency assay in two steps; first a reduction followed with a replacement by an Immunochemical assay as foreseen for TBEV vaccines. Beside the ethical gains of 3R it also allows to release vaccines faster, with less variable methods and in a leaner procedure. This displays that 3R is a win-win for patients, regulators and manufacturer.

While continuing to work toward non-animal-based research and development, GSK remains committed to a culture of care acting ethically and practicing good animal welfare when animal use is still required.

#### Presentation: Oral



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## ***In vitro* to *in vivo* extrapolation for developmental toxicity potency of valproic acid analogues**

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The devTOX quickPredict assay (devTOXqP) is a human induced pluripotent stem cell biomarker-based assay developed as an alternative to animal tests to screen for developmental toxicity potential. The developmental toxicity potential (dTP) concentration from the devTOXqP assay indicates a chemical's developmental toxicity potency. Previous work showed that the potency ranking of dTP concentrations for valproic acid and its analogues was consistent with *in vivo* developmental toxicity. In this study, we applied *in vitro* to *in vivo* extrapolation (IVIVE) to address whether the devTOXqP dTP concentrations could quantitatively predict the *in vivo* developmental toxicity lowest effect levels for these chemicals. We evaluated the impact of *in vitro* kinetics, pharmacokinetic (PK) parameters, and different PK models on IVIVE outcomes. To evaluate the effect of *in vitro* kinetics, an equilibrium distribution model was applied to devTOXqP assay to translate nominal concentrations to free and cellular concentrations, which were used subsequently in IVIVE analyses. A one-compartment PK model, standard physiologically based pharmacokinetic (PBPK) models, and pregnancy-specific PBPK models were used for reverse dosimetry. The equivalent administered doses (EADs) that would result in maternal or fetal blood concentrations equivalent to *in vitro* activity concentrations were estimated by IVIVE. These EADs were compared to lowest effect levels in rat developmental toxicity studies and/or human therapeutic doses, and to EADs from a recent OECD case study publication derived using different sets of *in vitro* data. We also explored a read-across approach incorporating structural similarity information in data interpretation. Our preliminary results showed close agreement between EADs and *in vivo* rat lowest effect levels, indicating that the devTOXqP assay can quantitatively predict developmental toxicity potential of chemicals at relevant concentrations.

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**Presentation:** Oral

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## **Application of newly validated route-specific *in vitro* genotoxicity assays to support the safety assessment of cosmetic ingredients**

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New *in vitro* skin models have been established as follow up assays to improve the prediction of potential genotoxicity of cosmetic ingredients in the absence of *in vivo* data. The reconstructed skin micronucleus test (RSMN) and RS Comet assays are now validated and have been accepted into the OECD test guideline development program. Here, we demonstrate their application to safety assessment by conducting a case study based on the oxidative hair dye, 4-nitro-1,2-phenylenediamine (B24). The strategy is based on an endpoint-triggered follow up of positive results from the Ames and *in vitro* micronucleus (MNvit) 2-test battery. For topically applied chemicals, the RSMN assay is recommended for MNvit positive chemicals, whereas, Ames positives should be tested in the RS Comet assay. B24 was positive in the Ames assay but negative in the MNvit assay; therefore, it was tested coded in the RS Comet assay. In experiment 1, B24 was non-cytotoxic and did not induce DNA breaks in keratinocytes or fibroblasts up to the lowest precipitating dose (50 mg/cm<sup>2</sup>). In experiment 2, in the presence of the repair inhibitor, aphidicolin, there was no statistical increases in %tail DNA that exceeded the historical controls up to 50 mg/cm<sup>2</sup>. B24 was concluded to be negative, which is in accordance with negative results in the HPRT mammalian cell gene mutation assay. The conclusion from this case study is that while B24 causes mutagenicity in bacteria, it is not genotoxic in mammalian cells or in a higher tier assay using human skin equivalents. The RS Comet assay allows an assessment under the relevant exposed conditions, i.e., topically, and in a test system containing relevant metabolizing enzymes. Further case studies are under way to evaluate other scenarios from the testing strategy.

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**Presentation:** Oral



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## Building trust in stem cell models for compound selection in pharma; a task worth the effort

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The discovery of the Yamanaka factors has opened the door to new innovative human-based stem cell models that could be applied in the drug candidate selection cascade within the pharmaceutical industry. Obviously, the promise of using human-based stem cell models is to improve translation of preclinical to clinical effects. What experience tells us, is that despite their human make-up, today's stem-cell models have some limitations. To name a few, cell maturation is a challenge and 2D morphology in a dish does not fully reflect human 3D organ physiology. There are many ongoing initiatives that are trying to overcome these limitations in the future. Irrespective of this, the current surrogate models can have value in a screening cascade if one appreciates the pros and cons! For the use of cardiac safety evaluation, we optimized and characterized a fluorescent dye mediated calcium transient assay, using an extensive set of clinically known compounds and evaluated the predictive value of the readouts (Van Ammel et al., 2019). Second to that we developed a system, that integrates the different readouts from the model into a novel hazard score for potential risk for cardiac side effects (Kopljar et al., 2018). Although, this hazard scoring system requires assay specific statistical tolerance interval analysis and readout specific weight factor settings, this method greatly facilitates the compound selection process in an objective manner in R&D and largely reduces the use of animals.

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**Presentation:** Oral

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## Bioprinting of humanized liver and lung models for infection studies

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Generation of bioprinted 3D organ models is a new powerful approach to replace animal models. By using human cells, humanized organ models can be produced that provide valuable insights of physiological and pathological processes relevant to human patients (Weinhart et al., 2019). A bioprinter using pneumatic extrusion technology was used to produce a humanized lung model (Berg et al., 2018). The alveolar epithelial cell line A549 was printed in a bioink consisting of alginate, gelatin and Matrigel. We demonstrate that the organ model is suitable to support viral replication of influenza viruses and may serve as tool to study infection biology. To the best of our knowledge, this was the first published study using bioprinted organ models for infection studies.

More recently, we improved the lung model. It now consists of a multicellular, bioprinted layer of human lung fibroblasts on top of which an alveolar epithelial cell line is seeded. Furthermore, macrophages were implemented into the model. The model is currently used to develop inhibitors for the SARS-coronavirus-2.

In a second approach, we generated a liver model consisting of HepaRG cells. The bioink contained human extracellular matrix and resulted in a liver model with high cell viability and desirable physiological parameters. The liver model was infected with human adenovirus 5, which causes severe hepatic infections in immunocompromised patients. In addition, we demonstrated that the bioprinted liver model is suitable to study transduction efficiency of adeno-associated virus (AAV) vectors (Hiller et al., 2018).

The presentation will also comprise work in progress on establishing clean bioprinting, i.e., the attempt to produce 3D organ models which are complete devoid of animal components such as FCS, Matrigel or gelatin (Berg and Kurreck, 2021). To this end, cells must be adapted to FCS-free media and new bioinks must be developed.

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**Presentation:** Oral



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## Genetically altered animals (GAA) – Why the Three Rs are important

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The legislation in the European Union protects, and sets out care, use and accommodation standards for, animals destined for scientific procedures. The legislation makes specific reference to the creation and maintenance of genetically modified animal lines in its definition for a procedure.

In 2017, around 30% of the 9.38 million animals used in the EU for research and testing were genetically altered, with the proportion as high as 64% for zebra fish and 36% for mice. In addition, a further 1.3 million animals were used in the creation and breeding of genetically altered animal lines, and 6.1 million genetically altered animals were reported as surplus in 2017 (EC, 2020a,b).

In the light of these numbers, it is clear that the requirements for a systematic application of the Three Rs are equally relevant for activities in relation to the creation and maintenance of genetically altered animals. However, gene-editing may by its nature pose specific issues that are not applicable for conventional animals. It is important, therefore, that due consideration is given to genetically altered animals from the choice of methods for gene-editing and tissue sampling to care and accommodation requirements, which may differ greatly from those of conventional animals.

Gene-editing, by design, may result in intended adverse effects. It is therefore essential that information of any such effects and measures to limit these, such as by the environment or the use of humane end-points, is submitted with the animals to the institute where animals are planned to be used. Finally, there should be on-going efforts to limit and reduce surplus both from the perspective of colony management, improved anticipation of research needs and the reduction of non-desired genotypes.

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**Presentation:** Oral

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## Promoting transparency around animal research across regions

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Transparency in the scientific use of animals is essential to provide reassurance for the adherence to the commonly agreed ethical and socially acceptable animal care and use standards. It is a prerequisite for developing trust between the animal use communities and society, and it is a key to making available the necessary facts and data for informed decision-making, be it for policy development, research funding or simply to understand the *status quo*.

Accordingly, improved transparency was set as one of the key objectives for Directive 2010/63/EU. As a result, Member States publish non-technical project summaries of authorized projects using animals. Statistical reporting requirements on animal use were revised and more complete and comprehensive data are now available annually across the EU.

However, recently, the bar has been raised even higher. Regulation (EU) 2019/1010 on environmental reporting, adopted in June 2019, and the related Commission Implementing Decision 2020/569/EU, adopted in April 2020, have introduced further requirements to make information available in a timelier manner, and with access to all interested. Europe is pioneering a new level of transparency – one of the key tools to allow pursuing alternatives where these are most needed.

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**Presentation:** Oral

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## Animal welfare body – Tasks and role

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The Three Rs, to replace, reduce and refine the use of animals in science, must be given due consideration and implemented systematically when animal use is proposed. As part of the instruments to implement this requirement, Directive 2010/63/EU(1) requires that establishments (user, breeder and supplying establishments) set up and maintain an Animal Welfare Body (Articles



26 and 27 of Directive 2010/63/EU). Member States may allow small users, breeders and suppliers to fulfil the tasks of the animal welfare bodies by other means.

Furthermore, even if the responsibility and foundation for a good culture care rests with everyone involved in the care and use of animals, the animal welfare bodies provide the key structure in developing and maintaining a good culture of care and bringing the Three Rs to life in the day-to-day activities of the establishment.

Animal welfare bodies are expected, within the establishment, to provide advice on the welfare and care of the animals, the application of the Three Rs, review management and operational processes, follow the development and outcome of projects, and advise on rehoming.

To facilitate a uniform approach to the implementation of the legislation, an Expert Working Group was convened to address *inter alia* the structure, composition and tasks of animal welfare bodies. National Contact Points of the Member States of the EU endorsed results of this work in a document on Animal Welfare Bodies and National Committees (European Commission, 2014)). It provides further guidance on the structure, composition and tasks of animal welfare bodies, and discusses the ways to achieve an effective animal welfare body.

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**Presentation:** Oral

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## Replacing the Draize eye test: Impedance spectroscopy as a 3R method to discriminate between all GHS categories for eye irritation

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For the toxicological endpoint of eye irritation, the first alternative test systems based on *ex vivo* or *in vitro* models have been developed and validated. However, besides all efforts, the Draize eye test is still not completely replaced by alternative animal-free methods because the alternative methods cannot distinguish be-

tween the globally harmonized system for the classification and labelling of chemicals (GHS) category 1 serious eye damage and category 2 eye irritation. To develop a single *in vitro* test to identify all GHS categories for eye irritation, we combined organotypic cornea models based on primary human cells with an electrical readout system that measures the impedance of the test model. By employing a non-destructive measuring system based on impedance spectroscopy, we could increase the sensitivity of the test system. Moreover, the impedance measurement allowed for the first time to detect the persistence of irritative effects by repeated measurements in an *in vitro* model and thus to distinguish between all GHS categories. Substances that do not need to be labeled stayed above 60% normalized to the negative control. Category 1 substances reduced the tissue integrity after application below 6% and the effect did persist over a period of 7 days. Category 2 substances however, could be identified by a decrease below 60% after the application of a category 2 chemical such as ethanol and increased again above 50% after 7 days. Thereby, all GHS categories of eye irritation could be identified by repeated measurements over a period of 7 days. Based on a novel prediction model we achieved an accuracy of 78% with a reproducibility of 88.9% to determine all three categories of eye irritation in one single test (Lotz, C et al., 2018). This could pave the way to replace the Draize eye test.

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**Presentation:** Oral

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## Network integration and modelling of dynamic drug responses at multi-omics levels

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A crucial step in developing alternatives to animal testing is to uncover *in vivo* relevant mechanisms from heterogeneous *in vi-*



*tro* data. This can effectively be realized by integrating the molecular activities in networks of interacting molecules.

We have developed a resource, ConsensusPathDB, that integrates molecular interactions from multiple sources (Herwig et al., 2016). Part of the content is an integrated protein-protein interaction (PPI) network that is used as a scaffold for the integration of multiple omics data (Barel and Herwig, 2018). We present an approach that integrates dynamic proteomics and transcriptomics data and applies network propagation based on random walk with restart in order to identify disease-relevant network modules. We applied the approach to the problem of identifying mechanisms of toxicity from anthracycline anti-cancer drug treatments. Anthracycline-induced cardiotoxicity represents a cumulative systemic effect over time and refers to changes in myocardial functions for example in left-ventricular ejection fraction (LVEF) as well as cardiac stress responses.

Time-resolved anthracycline treatment was measured in iP-SC-derived 3D cardiac microtissues and dynamic changes in the proteome and transcriptome were charted. Data modelling retrieved a network module consisting of 170 interacting proteins such as mitochondrial proteins, proteins of the extracellular matrix and sarcomere proteins that are relevant for multiple cardiac disease processes. Furthermore, we identified 70% of the proteins in cardiac biopsies of patients and show that selected candidates correlate with clinical parameters.

We demonstrate that the network modelling approach is useful for i) integration of different omics data, ii) boosting functional content of omics data, iii) identifying modules of interacting molecules *in vitro* that are clinically transferable, and iv) complementing missing information from single omics layers, in particular proteomics.

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**Presentation:** Oral

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## A microphysiological system of human pancreatic islet microtissues and liver spheroids for modelling diabetes mellitus

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To date, animal models are a cornerstone for the investigation of complex multi-organ diseases such as type 2 diabetes mellitus. However, due to obvious genetic and physiological differences between humans and animals, these can only partly model pathogenesis and complications specific to humans. Therefore, we have developed an organ-on-a-chip model to study the interplay between pancreatic islets and the liver, two key organs involved in the impaired glucose homeostasis characteristic for diabetes mellitus.

We demonstrated that human pancreatic islet microtissues and human liver spheroids can be maintained in a common culture medium for up to 15 days. Liver spheroid functionality, as shown by albumin secretion into the culture medium, was stable over the entire culture period. Islet functionality was investigated by insulin release into the culture medium as well as by a glucose-stimulated insulin secretion of islet microtissues extracted from the chips at the end of the culture period. The analysis revealed that prolonged hyperglycemia only impaired islet functionality in islet single cultures while islet microtissues in co-culture remained functional. Islet-liver crosstalk was demonstrated by an *in vitro* glucose tolerance test measuring glucose and insulin concentrations in response to a glucose load. Insulin secreted in the co-culture increased glucose utilization by the liver spheroids accelerating the rate of glucose disappearance from the culture medium, while the liver single culture alone did not regulate glucose levels as efficiently. As glucose levels fell, the insulin concentrations in the medium decreased thus demonstrating a feedback loop between islet microtissues and liver spheroids, which reflects the functional human glucose regulation.

In the future, the established co-culture model could be used as a unique *in vitro* system for the study of type 2 diabetes mellitus pathogenesis as well as for efficacy testing of anti-diabetic drugs.

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## In house GIVIMP method validation as a key process for accelerating thyroid *in vitro* methods from bench to regulatory use

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*In vitro* method development under quality assured conditions with a clear focus towards regulatory usability would be a perfect concept, but still too often it is wishful thinking. The vast majority of *in vitro* cell and tissue-based methods described in academic publications are innovative and of applied use, have mechanistic relevance, but lack scientific and quality considerations envisaging regulatory acceptance. Hence, experimental design decisions and documentation prepared in the development process must be substantially amended for validation for regulatory purposes. For the presented EU-NETVAL thyroid validation study, this was the situation for many of the selected *in vitro* methods.

Even if quality assurance-like working habits and quality control strategies are more and more established in the academic field, dedicated guidelines to improve the situation for *in vitro* method development has been missing. Recently, as a joint effort by OECD, EURL ECVAM, a variety of regulatory authorities and experts from the industry and different member states,

a guidance document on the development and proper conduct of *in vitro* methods was released – GIVIMP (OECD, 2018). The OECD GIVIMP guidance document aims to disseminate critical *in vitro* method information (e.g., on roles and responsibilities in the process of method design, validation and guideline development) and practical guidance (e.g., test system characterization, method performance, choice of reference & control items, SOP design) to test method developers. GIVIMP forms a solid foundation to encourage at the national and international level further initiatives. This will help to increase number and readiness of developed methods in the future, to facilitate and support their translational use as 3R-relevant *in vitro* tests and to improve the chance for successful guideline development and regulatory use.

Motivation and benefits of GIVIMP implementation from the developers' perspective and respective examples from the ongoing EU-NETVAL study on thyroid disruption will be presented.

### Reference

OECD (2018). Guidance Document on Good In Vitro Method Practices (GIVIMP). *Series on Testing and Assessment, No 286*. OECD Publishing, Paris.

### Presentation: Oral

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## Global collaboration, harmonization and interdisciplinary efforts deliver mechanistic methods and integrated approaches for identifying human thyroid disruptors

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Concern over the potential for environmental chemicals to perturb hormone systems has led to the development and implementation of several OECD Test Guidelines for the screening and testing of endocrine disrupting chemicals. Although a number of methodologies have been developed to interrogate reproductive steroids, incorporation of test methods to evaluate disruptors of thyroid hormone signaling pathways has been limited, owing largely to the complexity of the thyroid system. Research efforts are funded by the European and International funding programs to support the necessary development of new methods and approaches in this field to complement the information gaps identified. Furthermore, the European Commission Joint Research Centre's European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) has compiled several Thyroid Hormone Disruption (THD) *in vitro* methods with validation potential, based on the OECD scoping paper (OECD, 2014) and with input from THD meetings and workshops. This large set of 17 methods has been used as the starting point for validation of selected *in vitro* THD methods for the identification of modulators of thyroid hormone signaling. Central to the validation exercise is the selection of the chemical validation set. A chemical expert group proposed a list of 51 chemicals that has been reduced to a final set of 30 chemicals informed by the use of chemoinformatics tools and Artificial Intelligence based (AI) tools, i.e., machine learning and text analytics. The symposium will illustrate how global collaboration, harmonization, interdisciplinary efforts and increasing common awareness of common agreed regulatory information needs can deliver methods and approaches responding to current regulatory challenges for identifying human thyroid disruptors.

#### Reference

OECD (2014). New Scoping Document on In Vitro And Ex Vivo Assays for the Identification of Modulators of Thyroid Hormone Signalling. *Series on Testing and Assessment, No. 207*. OECD Publishing, Paris.

**Presentation:** Oral

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## Towards the replacement of fetal bovine serum in cell culture application: The example of A549 cells

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Fetal bovine serum (FBS), used as a supplement for cell culture media, presents significant scientific concerns, such as the presence of undefined components and batch-to-batch variation.

The aim of this study is to explore how to transition cells to FBS-free media, using the alveolar epithelial type II A549 cell line as an example. The final goal is the complete replacement of animal derived components for cell culture application in A549 cells, which is commonly used in respiratory toxicology testing.

A549 cells were transitioned to different commercially available FBS-free media (i.e., XF212, HL-1, XVIVO, CNT-PRA, PneumaCult). After successful transition to FBS-free medium, different strategies were used to freeze cells (i.e., using medium containing 20%FBS + 10%DMSO, 10%DMSO only, 7.5%DM-SO + 10%cell culture-grade bovine serum albumin or Profreeze and CNT-CRYO-50 freezing media). Of the tested options, the Profreeze freezing medium showed the best cell survival rates when freeze-thawing the cells.

A549 cells did not survive the direct replacement in any of the media; however, a gradual replacement (according to suppliers' instructions) was successful for XVIVO and CNT-PRA media. After several passages in FBS-free conditions, A549 cells displayed different cell morphology (e.g., larger cells) dependent on the media used. The growth of the cells was evaluated and the doubling time of A549 cells is more than two times higher in alternative media compared to the control with 10% FBS. Toxicity testing (cytokine analysis) is ongoing to determine if these differences in morphology and growth rate effect the functionality of these cells and if the use of a chemically defined medium allows for the generation of more reproducible results.

It is possible to replace animal derived components for the culture of A549 cells, which represents a considerable advance in the reproducibility of cell culture and that could be applied to many other cell lines.

**Presentation:** Oral



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## Safety sciences towards non-animal tests in China: Current status and perspectives

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With the recognition of the importance of 3Rs (Replacement, Reduction, and Refinement) in science and regulation, China has been undergoing a gradual shifting toward 21<sup>st</sup>-toxicity testing paradigm. The newly effective Cosmetics Supervision and Administration Regulation (CSAR) and its subsidiary regulations have marked shifting of safety assessment toward ingredient-based and exposure-oriented practice. This lays the foundation for more widespread recognition and use of contemporary non-animal testing approaches, such as *in vitro* toxicity tests, computational modelling, and integrated approaches. Increasing alternative methods has been adopted in the Safety and Technical Standards for Cosmetics (STSC, 2015 version) since 2016. The recently issued Measures for the Environmental Management Registration of New Chemical Substances by MEE (Ministry of Ecology and Environment) has further harmonized with international practice that increases acceptance of alternative methods and outlines several exemptions. The new regulation frameworks are expected to further promote the upgrading of the regulatory system and industrial engagement.

Continuous academic efforts have also pushed the boundaries forward. The Society of Toxicological Alternatives and Translational Toxicology under the Chinese Society of Toxicology and the Society of Toxicity Testing and Alternative Methods under the Chinese Environmental Mutagen Society have provided a scientific communications interface for government, enterprises, and academia via national/Asian conferences, training, and other activities. In 2018, alternative development proposal was included in the latest national strategic document released by the China Association for Science and Technology. Monographs that addressed 21<sup>st</sup>-century toxicity and *in vitro* toxicology was published or to be published. It is foreseen that multidisciplinary and cross-regional collaboration will further promote the translation of modern toxicology in China.

**Presentation:** Oral

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## Building an internal information infrastructure to disseminate surplus animals

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The Research Facilities for Experimental Medicine (FEM) of Charité Universitätsmedizin Berlin manage their animal population with an online database. This database records all animals until they are no longer kept. Since the FEM also breeds its own lines, both breeding stock and experimental stock are documented. The number of so-called surplus animals is correspondingly high.

In accordance with the 3Rs, the number of animals should be kept to a minimum. The FEM therefore endeavors to ensure that animals which cannot be used in animal experiments to be passed to other working groups. Up to now, the transmission of animals at the experimental sites has been done orally or by internal circular mail. Then a solution with the database could be found and the FEM animal pool was established. At first this was also done at the experimental sites by animals given by researchers. In the meantime, the animal pool has been extended to the breeding sites, so that breeding animals can also be placed. Researchers and animal caretakers can use the database to transfer surplus animals in the animal pool and also retrieve animals from the pool for their projects. In the sense of the 3Rs, researchers are requested to check whether animals from the pool can be used for their project before ordering animals externally.

In this presentation, the results of the currently ongoing test phase, the procedure, the animals called up, internal and official limitations are presented.

**Presentation:** Oral

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## Towards building harmonized and interoperable e-infrastructures for reproducible new approach toxicology – The OpenRiskNet concept

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The paradigm shift in risk assessment from descriptive, animal-test-based to mechanistic new approach methods and especially the introduction of the adverse outcome pathway (AOP)



concept has resulted in the need for advanced protocol and data management concepts, access to a multitude of harmonized data sources and *in silico* tools to process all the different pieces of evidence combining toxicodynamics and biokinetics. We will present here new developments specifically targeting these demands performed in different projects funded by the European Union in the chemical and nanomaterial safety area.

Providing data, protocols and software services via independent, semantically annotated application programming interfaces opens up new ways of assessing and producing all relevant information and integration into automated and reproducible risk assessment workflows. The infrastructure projects OpenRiskNet (<https://openrisknet.org/>) and NanoCommons (<https://www.nanocommons.eu/>) are providing knowledge platforms and data warehouse concepts giving access to data from different European, e.g., EU-ToxRisk, as well as international projects, including, e.g., new access routes to the TOX21 and TG-GATEs datasets, in a curated, harmonized and interoperable way. These can be analyzed and combined with computational/modelling approaches also on the OpenRiskNet platform. To demonstrate the flexibility of this new concept, workflows will be shown using different data and knowledge bases and the semantically annotated and linked data therein to computationally generate key events and validate AOPs with experimental data re-usable, e.g., for the development of (quantitative) AOPs and read-across applications in the case studies of EU-ToxRisk (<https://www.eu-toxrisk.eu/>).

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**Presentation:** Oral

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## Applied and delivered dose determination for ALI acute inhalation toxicity testing of a petroleum-derived substance

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To reduce animal testing there is an increasing demand to implement *in vitro* alternatives for inhalation testing using human relevant lung cell models, realistic air-liquid interface (ALI) expo-

sure systems, and proper dosimetry techniques. Petro-chemical industry supports the shift from *in vivo* towards *in vitro* testing and provided funding to study the effects of inhalation exposure to petroleum substances.

ALI inhalation experiments using a single compound (ethylbenzene-EB) and a complex mixture (gasoline-GO) were performed. A generation facility was successfully developed to volatilize EB and GO. A capillary dosing unit was connected to a jar containing a heated base plate. The compound was vaporized and mixed with a humidified air flow. Subsequently the humidified vapor flow was cooled down and guided towards the climatic chamber (37°C) where 4-hour ALI cell exposure took place using the VITROCELL<sup>®</sup> 24/48 exposure system.

Applied dose was determined by combining microbalance consumption and airflows and verified using GC-FID and charcoal sorbent tubes. Delivered dose was determined using different sample preparation techniques. These techniques were compared to select the most suitable method for sample preparation of cell cultures exposed to volatile organic compounds. Cell culture samples and cell culture medium were analyzed using Head-space GC-MS.

For EB a significantly decreased mean cell viability of 86%, 77%, and 47% was observed for exposure of A549 cells to a applied dose of EB of about 30,000, 40,000, and 50,000 mg/m<sup>3</sup>, respectively. In a different experiment cells were exposed to a GO average concentration-range of about 5600, 8400, and 11,000 mg/m<sup>3</sup>. Generation up to a maximum of 11,000 mg/m<sup>3</sup> gave no effect on A549 cell viability. In both cases deposited dose was compared with *in vivo* data.

**Presentation:** Oral

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## Optimizing drug discovery by Investigative Toxicology: Current and future trends

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Investigative Toxicology describes the de-risking and mechanistic elucidation of toxicities, supporting early safety decisions in the pharmaceutical industry. Recently, Investigative Toxicology has contributed to a shift in pharmaceutical toxicology, from a descriptive to an evidence-based, mechanistic discipline. This was triggered by high costs and low throughput of Good Laboratory Practice *in vivo* studies and increasing demands for adhering to the 3R (Replacement, Reduction and Refinement) principles of animal welfare. Outside the boundaries of regulatory toxicology, Investigative Toxicology has the flexibility to embrace new technologies, enhancing translational steps from *in silico*, *in vitro* to *in vivo* mechanistic understanding to eventually pre-



dict human response. One major goal of Investigative Toxicology is improving preclinical decisions, which coincides with the concept of animal-free safety testing. Currently, compounds under preclinical development are being discarded due to the use of inappropriate animal models. Progress in Investigative Toxicology could lead to humanized *in vitro* test systems and the development of medicines less reliant on animal tests. To advance this field a group of 14 European-based leaders from the pharmaceutical industry founded the Investigative Toxicology Leaders Forum (ITLF), an open, non-exclusive and pre-competitive group that shares knowledge and experience. The ITLF collaborated with the Centre for Alternatives to Animal Testing Europe (CAAT-Europe) to organize an “Investigative Toxicology Think-Tank”, which aimed to enhance the interaction with experts from academia and regulatory bodies in the field. This talk highlights the start and the outcome of the joined activity on Investigative Toxicology.

**Presentation:** Oral

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## Implementation of the 3Rs in biomedical research

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With the EU Directive 2010/63 and the revision of the law for animal experiments in Austria 2012, the 3Rs (Replacement, Reduction and Refinement of animal experiments) became a crucial issue for all persons involved in animal experiments. The Division for Biomedical Research (BMF) of the Medical University of Graz divides the efforts of the 3Rs in five main pillars: 1. 3Rs in action is an internal platform of the Medical University of Graz stating that animals which are no longer used for a project or that had to be excluded are available to other researchers. During this time, the housing costs for these animals will be covered by the BMF. As a results numbers of animals at the Medical University of Graz have already been reduced and the acceptance of the 3Rs was strengthened. 2. Optimized animal husbandry by using innovative techniques. 3. Establishment of the Core Facility Alternative Biomodels and Preclinical Imaging with the focus on xeno-free cell culture, human autologous cell culture models and the establishment of a high-precision preclinical imaging center. 4. Development of an appropriate evaluated education/training platform with modular structures in the field of animal experiments and innovative alternative technologies. Finally, 5. Co-foundation and promotion of the RepRef Red society “*Gesellschaft zur Förderung von alternativen Biomodellen*” which is responsible for the organization of congresses, conferences, workshops and offers a platform for knowledge transfer in the field of alternative methods in Austria.

Animal welfare and animal ethics are fundamental topics for every researcher working with animals, and especially for animal facilities themselves. The implementation of the 3Rs is not primarily about replacing animal experiments, but for the time being about a greater reduction and refinement of current research.

**Presentation:** Oral

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## European network of high-quality laboratories assessing efficiently the relevance of fully animal-free thyroid mechanistic methods

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ECVAM set up a consortium of more than 30 European laboratories (mostly GLP approved), called EU-NETVAL in 2013, to use these for upcoming validation studies based on their competencies (technologies, technical equipment, scientific knowl-



edge). Part of this network was considered for the development and validation of *in vitro* methods to target different modes of action (MoA) of thyroid disruption.

In the first part, the developers of the selected methods were brought together with one EU-NETVAL lab each to define the method including SOP development, to assess robustness, transferability and within-laboratory reproducibility. In the second part, ~30 reference chemicals with known MoAs will be tested to define the relevance of each method. Depending on the available methods, the challenges for each MoA are being different: 1) available peer-reviewed papers are old and the developer is no longer available 2) test systems not commercially available or not standardized 3) reference items do not show the expected outcome 4) method is not user-friendly and need to be adapted 5) reproducibility of the method is not sufficient. Despite these challenges, in the first quarter of 2020 experimental work including generation of SOPs was ongoing for most method. There are also success stories, where the transfer of the methods was already successful. Beyond the technical challenges, a putative defined approach is still unclear and should be defined by considering the strengths and weaknesses of the *in vitro* methods under validation, the physiological feedback mechanism and the respective *in vivo* data sources (animals, humans).

The final goal of the project is to set up a robust and reproducible tool box of relevant *in vitro* methods to predict thyroid disruption in the framework of the ECHA/EFSA Guidance on identification of endocrine disruptors (ECHA et al., 2018) and other global regulatory needs.

#### Reference

ECHA, EFSA et al. (2018). Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009; *EFSA J* 16, e05311. doi:10.2903/j.efsa.2018.5311

**Presentation:** Oral

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## Digital twins for personalized healthcare

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A digital twin is a virtual representation of its physical counterpart, bringing together all relevant data of the physical part – preferably continuously updated with new data – and adding an intelligence layer on top of it to extract insights hidden in the data and predict future performance or issues. Whereas digital twins for devices or equipment are already known for quite some time in industries like Aerospace, Automotive and Energy, it's a relatively new concept in Healthcare. This is certainly true if we translate the digital twin concept to patients.

In this keynote we will dive into this concept of patient digital twin, discuss the current state and sketch a future vision on its applicability. We will look at how the patient digital twin can transform the healthcare industry, with applications in clinical space (for example in diagnosis, treatment selection, treatment planning and monitoring) but also in pre-clinical space (for example limiting the need for animal testing).

Apart from big promises there are also big challenges, so not only the vision and current status of developments will be discussed, but also the challenges that must be overcome and the limitations that we need to take into consideration.

**Presentation:** Oral

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## Complex *in vitro* models that could impact 3Rs in early drug discovery

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The pharmaceutical industry is continuing to face high R&D costs and low overall success rates of clinical compounds during drug development. There is a surge in demand for development and validation of disease relevant and physiological human cellular models that can be implemented in early-stage discovery, thereby shifting attrition of future failures to a point in discovery where the costs are significantly lower and where it could also impact animal usage for safety and pharmacological testing. The current drug discovery paradigm involves lengthy and costly lead discovery and optimization campaigns, often using simple cellular models or animal models especially in Immuno-oncology with variable translational relevance to human disease or safety. This illustrates a failure to effectively and efficiently reproduce human disease-relevant states at an early stage to steer target and compound selection before going to safety testing in animals, and then onto human clinical trials. Therefore, a fundamental question is, how do we recapitulate human biological complexity of a disease state in robust translational *in vitro* assays for interrogation to increase our success rate in late-stage drug discovery programs. The majority of new complex *in vitro* technologies that promise to be influential and more predictive in the next few years need to be qualified and customized for drug discovery efforts. Examples of time-dependent, multiplexed and multi-dimensional 3D cell culture approaches (eg spheroids, organoids and microphysiological systems) used to create more patient centric models in both safety and efficacy to better characterize targets and progress molecules earlier in discovery will be described. These case examples will be used to elaborate on the importance and methodology to qualify these complex *in vitro* models and the translatability of these models to the clinic. Finally, opportunities will be covered wherein we aspire to combine



computational modelling, AI/ML with Complex In Vitro Models (CIVMs) and ways to speed up the implementation/adoption process of CIVMs through working with regulatory bodies or interactions with organ-on-a-chip networks, 3Rs organizations, and consortia such as the IQ-MPS affiliate, or other Microphysiological systems organizations.

**Presentation:** Oral

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## The animal protection quality certificate. Key figures for the improvement of animal welfare

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The 3R concept was developed in 1959 by Russel and Burch to banish inhumanity from animal testing. After extensive discussion, these principles were finally implemented in the EU Directive 2010/63, which imposed the obligation to implement them into national legislation of the member states. This signifies the demonstrable documentation of 3R procedures used in practice. The 3Rs are including Replacement and Reduction of animal experiments *per se* as well as Refinement of the methods applied in animal experiments and in the husbandry and care of animals in order to reduce animal suffering to a minimum. The last years have shown that communication about direct Replacement methods is more popular and has a longer tradition than discussions about Refinement.

This could be due to a better measurability of the success of Replacement methods. However, it is more difficult to prove the effects of Refinement on animal studies. Despite urgent efforts to replace or at least minimize animal experiments, Refinement is the only way to ensure the welfare of animals during an experiment. The improved methods in husbandry and experimentation automatically lead to a Reduction in the number of animals in individual experiments.

The Austrian 3R Initiative (RepRefRed Society) implemented a tool for assessing the refinement Methods via the Quality Management System of an animal facility. With the implementation of the right key figures improvement of Refinement strategies during housing and during experiments are easily to assess. This till will improve the animal welfare in animal research facilities during housing and during animal experiments.

**Presentation:** Oral

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## Seeing with 2020 vision the future of chemical safety

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The current view of chemical safety suggests that challenges remain largely the same – tens of thousands of chemicals are currently in commerce, and hundreds more are introduced every year with only a small fraction of chemicals fully evaluated for potential human health risks. The scientific community has struggled with integrating technological and computational advances to address these challenges due to the potential public health and regulatory implications of the underlying science, differences across regulatory jurisdictions and societal pressures, and the lack of clear standards for success. The endpoints and uncertainties associated with these new technologies are qualitatively and quantitatively different than the traditional approaches. A 2020 vision for chemical safety requires a multi-disciplinary effort across toxicology, exposure science, and chemical risk assessment to bridge this gap and more effectively translate advances to regulatory decisions. The talk will highlight progress in these areas and provide a path to success for protecting public health and the environment. This abstract does not necessarily reflect U.S. EPA policy.

**Presentation:** Oral

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## Multi omics data integration to study the relevance of *in vitro* disease models through the creation of genomic interaction networks

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*Introduction:* The integration of different omics data sets can provide a more holistic view of *in vitro* disease models. This could help to identify pivotal features on a molecular level and therefore might be a potential tool to deeper understand cellular mechanisms and assess their relevance for the human *in vivo* situation instead of solely investigating classical endpoints (Kroeger, 2006). Here, we propose a multi-layer Nonnegative Matrix Factorization (NMF) to detect both inter- and intra-relationships in all layers of omics information to extract signature profiles, which can be studied with genomic interaction networks.



**Method:** The multi-layer NMF method clusters the samples in a data set based on the combination of different omics measurements. For each cluster, a genomic interaction network is built that contains information about gene – gene interactions, CpG site – gene interactions, compound – gene interactions (Comparative Toxicology database) (CTD, 2017) and disease-gene associations (DisGeneNet) (Piñero et al., 2019).

To evaluate the proposed workflow, we used DNA methylation and gene expression data from the 2019 Cancer Cell Line Encyclopedia (Ghandi et al., 2019), DNA methylation being a form of epigenetic control that plays an important role in gene regulation.

**Results:** For the 2019 Cancer Cell Line Encyclopedia, we have identified eight clusters that contain different gene and CpG signatures. From these signatures, the genomic interaction networks are built to study gene – gene and CpG – gene interactions. Here, we focus on one cluster that contains only lymphoid neoplasms. A set of methylated genes are identified that could propagate their effect towards interacting genes, and might play a role in the onset of lymphoid neoplasms. Future case studies can be applied to investigate further *in vitro* disease models through the obtained genomic interaction networks, which could help to identify exposure-related signatures on the omics layers.

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**Presentation:** Oral

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## Biokinetics and dose-response models to predict thyroid disruption effects

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In order to make logic out of *in vitro* measurements for regulatory decision-making efforts are undertaken to extrapolate from the *in vitro* situation to *in vivo* by developing biokinetics and dose-response models to predict thyroid disruption effects. An example of such a complex kinetics and dose-response model to predict thyroid function disruption due to iodine deficiency and exposure to thyroid-active chemicals in near-term pregnant woman has been developed by Lumen et al. (2013). The model includes a physiologically based pharmacokinetic (PBK) pregnancy model for iodide and perchlorate and sub-models for thyroid hormones T4 and T3. Efforts are undertaken for a global harmonization on the use of PBK models in regulatory decision-making (Sachana, 2019).

The Lumen model allows for the prediction of internal target-tissue specific concentrations of iodide, perchlorate, and thyroid hormones based on external administered dose, pharmacokinetics, and mode of action of iodide and perchlorate. This first-of-its-kind model was used by the U.S. EPA and U.S. FDA as a proof-of-concept model in the regulatory evaluations of perchlorate in food and drinking water (EPA, 2017; FDA, 2017). The model was further extended to a population level dose-response model and captures severe iodine deficiency conditions incorporating the effects of feedback mechanisms of the hypothalamus-pituitary-thyroid axis (Lumen et al., 2015, 2017a,b). The population model was used for reverse dosimetry applications using the available biomonitoring data of urinary iodide concentrations to reconstruct the iodide intake distribution (Lumen et al., 2017a). Inclusion of additional modes of action such as TPO inhibition and activation of thyroidal passive permeabilities showed improvements in predictions (Willemin and Lumen, 2016, 2017, 2019). PBK modelling, mode of action-based interpretation and reverse dosimetry are a key tool for a real paradigm shift in regulatory evaluations of thyroid-active chemicals.

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**Presentation:** Oral

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## Overview of exposure-led, hypothesis-driven animal-free safety assessment of cosmetics

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The AFSA Cosmetics education and training program was created to facilitate global capacity and confidence building in cosmetic safety assessment without the generation of new animal testing data. The program follows the International Cooperation on Cosmetics Regulation (ICCR; comprised of regulators and scientists from Brazil, European Union, Japan, and United States) principles and is based on the “next generation risk assessment” workflow designed in the European Union Framework Program 7 project Safety Evaluation Ultimately Replacing Animal Testing (SEURAT-I). The AFSA project has organized the NGRA process into 8 modules for development: Problem Formulation, Consumer Exposure, In silico Tools, Exposure-based waiving, Internal Exposure, In vitro Data Synthesis, and the Overall Risk Assessment. The modules are being developed for product developers and evaluators that have different levels of experience with the NGRA process. My talk will introduce NGRA and the project modules and how they fit together to provide comprehensive information on animal-free safety assessment of cosmetic products and ingredients. This symposium presents sample elements of these modules: exposure-based safety assessment of cosmetics, application of *in silico* models, an endpoint-specific case study and wrapping up with a presentation on bringing it all together – integration of new approach methodologies for cosmetic safety decision making.

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**Presentation:** Oral

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## Mini Me – Tissue-on-a-chip as a mimic for patient response

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In the last 20 years, lab-on-a-chip (LOAC) has become a reality. Microlitre volumes of fluids can be accurately flowed over specific areas of a device that can contain precisely patterned arrangements of cells, engineered tissues or patient biopsies. An important facet of the LOAC approach is that analytical systems can be reduced to a similar scale so they too can be integrated onto the same platform (Whitesides, 2006) allowing accurate and precise characterisation of metabolites/biomarkers of interest.

In 2012 the National Institute of Health’s partnership with the Defense Advanced Research Projects Agency and the US FDA, facilitated a step change in LOAC research by commencing a \$70 million, 5-year plan, supporting 17 projects. The principal aim of this initiative was to develop new models for the efficient screening of drugs in a cost-effective manner by reducing attrition and overall drug pipeline costs (NCAT, 2019).

An additional advantage of the organ-on-a-chip (OOAC) approach is that it has the potential to reduce and, in some instances, replace animal models. The multidisciplinary group in Hull have been working on designing, fabricating and characterizing devices, made from either glass or polymers that can maintain fresh tissue biopsies in a functional state since 2008. Biopsies from human tissues, including head & neck squamous cell carcinoma, glioblastoma, thyroid and colorectal carcinoma, normal liver and heart have all been studied. These samples have been subjected to different chemotherapeutic drugs and irradiation regimens with response being monitored using a variety of analytical techniques (Riley et al., 2019; Kennedy et al., 2019).

For widespread adoption of OOAC for drug screening and a personalised therapeutic selection tool, it must mimic the human system better than the current 2D, 3D or animal models. The



presentation will showcase studies attempting to demonstrate where the tissue-containing devices begin to fulfil these criteria.

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**Presentation:** Oral

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## Scientific workshop on non-animal approaches for chemical safety in China: Current progress and outlook

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In collaboration with pioneering Chinese scientists across regulatory laboratories, research institutes and universities, Unilever organised a scientific workshop on non-animal approach-

es for chemical safety in Shanghai on 10<sup>th</sup> and 11<sup>th</sup> April 2019. Like the rest of world, significant scientific progress has been made over the last decade in China on non-animal approaches to chemical safety. In particular, the use of new approach methodologies (NAMs) for making consumer safety risk assessment decisions was reviewed in light of recent advances in this area as summarised by the International Cooperation on Cosmetics Regulation (ICCR; Dent et al., 2018). The first day of the workshop reviewed developments in *in vitro* models in China (including organ-on-a-chip technology and 3D bio-printing) as well as advances in omics technologies (including dose-dependent transcriptomic approaches for screening and prediction of chemical toxicity) and computational modelling. There was also discussion of regulatory developments and approval of alternative methods in China (including recent adoption of OECD non-animal methods into Chinese technical guidance). The workshop then focussed on the future of non-animal approaches for chemical safety in China with breakout groups identifying needs for the future in 5 defined areas (1) *in vitro* models (2) *in silico* models (3) regulatory science (4) education and training and (5) application in next generation risk assessment. Several opportunities were identified to accelerate the scientific and regulatory advances in adoption of non-animal approaches for chemical safety in China including opportunities through global international partnerships with global scientific programmes.

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**Presentation:** Oral

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## Breaking the lock-in to animal research within academia

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New approach methodologies (NAMs) are developing in leaps and bounds, yet the rate at which they are being adopted to replace the use of animals in research is frustratingly slow. The problem of “lock-in” to animal research is often referred to in this context, following Frank (2005) who first applied the concept to this field.

I consider our cultural attachment to animal research, as well as some of the key features that lock us into the paradigm, including aspects of animal research regulation and current efforts to improve the quality of animal studies. Insights from the philosophy of science (e.g., Kuhn, 1962; Lakatos, 1970; Laudan, 1977) suggest that the slow rate of change and the types of challenges faced by those attempting to replace animal research with NAMs are typical for a scientific revolution. I consider how we can use



insights from the philosophy of science, as well as research into the diffusion of innovations, to overcome some of the barriers, break the lock-in to animal research and hasten this particular scientific revolution.

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**Presentation:** Oral

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## Safety testing of biologics with an organs-on-chip platform: Lessons learned from the unique Roche Emulate partnership

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T-cell bispecific (TCBs) antibodies are an innovative tool for harnessing the immune system to fight cancer. TCBs simultaneously bind to a T-cell and a tumor antigen resulting in tumor killing. TCBs efficacy relies in part to the ability of the T-cell to access the tumor tissue, while their safety is driven by levels of expression of the tumor antigen in normal tissues. Traditional preclinical safety assessment is challenged by the poor translatability of animal to human immune responses. In addition, expression levels and localization of target antigens often differ between species and are rarely recreated in conventional *in vitro* models. Therefore, our aim was to develop complex human cell-based models that could better predict off-tumor, on-target TCB toxicities. In this presentation, we will describe the joint research

strategy by Roche and Emulate to develop an Alveolus Lung-Chip model utilizing primary epithelium cultured with endothelial and immune cells. Our strategy has benefited from clinical and non-human primate findings with different Roche TCBs to optimize the predictive potential of the models and to validate them through back translation. Additionally, this approach allowed us to elucidate potential mechanism of action of TCBs for prediction of unforeseen safety concerns and improve human risk assessment.

**Presentation:** Oral

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## KCs, MOAs and AOPs – Mutually informative, not mutually exclusive

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Adverse Outcome Pathways (AOPs) were conceived as a framework to organize data and help inform decisions regarding chemical safety. Since 2012, OECD had provided a platform for AOP information and development, and though they are now part of modern toxicology, the use of AOPs for regulatory decisions is still limited. Other strategies exist for organizing and evaluating toxicological data, such as Mode of Action (MOA) and Key Characteristics (KC) approaches, which require a less thorough understanding of the biological sequence of events linking chemical exposure and the apical effect on organisms and do not impose a linear relationship on complex physiological interactions. OECD is reconsidering how to bridge the substantial gap between AOP development and regulatory application. In the examples provided in this session, there are varying levels of understanding of the events linking a molecular initiating event to an adverse outcome, yet each example can be used in a regulatory setting. Furthermore, each example can be overlaid onto an AOP framework and used to identify gaps in biological understanding, develop tools to measure intermediate effects, and aid development of predictive models. At first glance, these may appear to be competing approaches for organizing data, but we propose they can be mutually informative and may be deployed for different purposes. For example, KCs may be used to search for available data and also viewed as molecular initiating events or early key events in AOPs. Ultimately, something less than a complete AOP may be perfectly adequate for reproducible, reliable prediction of an *in vivo* outcome and reduce the need for animal testing for some types of toxicity.

**Presentation:** Oral



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## Meningococcal group B vaccine: A journey towards a complete animal test free release process

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Meningococcal disease is caused by a gram-negative diplococcus, *Neisseria meningitidis*. 4CMenB is a Meningococcal group B vaccine indicated for active immunization to prevent invasive disease caused by serogroup B.

According to the European Pharmacopoeia and the Code of Federal Regulation, the release process established a series of quality control tests to guarantee its potency, purity, safety and pyrogenicity. The following *in vivo* tests have been part of the release testing panel: (RPT) Rabbit Pyrogen Test; (ATT/GST) Abnormal Toxicity/General Safety Test; (MDRP) Multi Dilution Relative Potency.

In accordance with the company's commitment to 3Rs principles, we focused on the waiver of those tests without any added values (Reduction), and on moving testing process from *in vivo* to *in vitro* (Replacement), with the final purpose to achieve the complete replacement of animals use.

From 2010, European Pharmacopoeia allows use of MAT (Monocyte Activation Test) to replace RPT, and in 2013, GSK started collaboration with NIBSC for development of this assay. MAT test is now licensed to release our vaccine in almost all markets.

Approval by CBER of removal of the GST for US market was also possible due to demonstrated consistency of the manufacturing process and due to the FDA decision to remove the test.

Finally, we are about to request for approval an in-house developed IVRP (In Vitro Relative Potency) assay. This ELISA test is intended to substitute the currently approved *in vivo* potency assay. IVRP method has been successfully validated and will be submitted soon to Health Authorities worldwide.

Although the set-up and validation of the new *in vitro* methods have been particularly demanding, GSK has maintained focus on its 3Rs program and we are confident that in due time we will be able to obtain the final approval for the remaining *in vitro* methods, to complete successfully our journey.

**Presentation:** Oral

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## Bringing it all together – Integration of new approach methodologies (NAMs) for cosmetic safety decision-making

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Exposure-based approaches and hypothesis-driven data generation form the basis of non-animal cosmetic safety assessments. These approaches are often described as New Approach Methodologies (NAMs) and can include computational models and human-relevant *in vitro* assays which are applied in combination to provide information on ingredient hazard and risk assessment.

Established approaches such as physiologically based kinetic (PBK) modelling, exposure-based waiving, predictive chemistry, History of Safe Use approaches and internationally accepted (OECD) *in vitro* methods enable the risk assessment of many ingredients in the context of how individuals are exposed to them within normal use. However, for more novel, higher exposure materials bespoke assays are required to understand biological effects from both a targeted perspective to ensure identification of the most sensitive effects, and broader screening approaches to ensure adequate biological coverage. The ability to confidently determine adequate margins of safety are essential to ensure the protection of consumers.

In this session, we have heard how external exposure is assessed, how PBK modelling informs internal exposure estimates, and have seen examples of NAMs for cosmetic safety. Here we present end-to-end case studies based on a non-animal risk assessment framework. In-use exposure scenarios will be used to illustrate how NAMs are applied in a hypothesis-driven ab initio approach to assure the safety of ingredients in consumer goods. Consideration will be given to assumptions and sources of uncertainty in the risk assessment and how they contribute to decision making.

**Presentation:** Oral

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## 3Rs approach for potency testing of human combined DTaP vaccines: Current status and next steps

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Human vaccines containing diphtheria (D), tetanus (T) and acellular pertussis (aP) antigens are still currently tested for potency with assays entailing the use of laboratory animals. This testing is per-



formed in the frame of mandatory quality control which implies for vaccine manufacturers to comply to different regulations applicable worldwide and not necessarily harmonized. Current methods are either challenge assays (evaluation of level of protection after vaccination followed by challenge with the pathogen) or serological assays (evaluation of level of antibody production after vaccination). The serological assays allow to achieve refinement (no clinical signs or death inflicted to animals) and reduction in the number of animals used when applying a single dilution assay.

As a further step in 3Rs, Sanofi Pasteur is currently implementing a single-dilution immunogenicity assay (SIA) allowing for a simultaneous assessment of potency for all antigens. The assay, its readout and its associated challenges will be described in the presentation.

Furthermore, a full *in vitro* approach to evaluate D, T and aP potency is being developed. This new *in vitro* testing approach will be able to reflect both antigen content and functionality, detect antigen alteration (stability indicating) and antigen differences relevant for production process. It will ensure the consistency of the product and maintain the link with batches found efficacious through clinical studies and routine use. As ultimate goal, it will allow to completely remove the use of animals for quality control potency testing for human D, T, aP containing vaccines once hurdles for worldwide regulatory acceptance have been over-crossed.

**Presentation:** Oral

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## What is truly animal-free testing? Moving towards animal-product-free *in vitro* systems

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Animal testing on cosmetics was banned in Europe in 2013, with similar bans following in other regions, but most “non-animal” methods still use a variety of animal-derived components and therefore cannot be described as truly animal-free. One major concern is the ongoing, widespread use of foetal bovine serum (FBS) in cell culture, even for human cell lines. Since FBS is derived from blood extracted by cardiac puncture of living, unanaesthetised calf foetuses, it has been questioned whether this process should be re-classified as a live animal procedure (van der Valk et al., 2017). At XCellR8, we have developed a 7-point scale that classifies test methods according to the level of animal suffering involved. Level 1: *in vivo* testing. Level 2: *in vitro* with test components that involve live animal suffering (e.g., FBS). Level 3: *in vitro* with test components that require “humane” animal sacrifice (e.g., rat liver extract). Level 4: *in vitro* with test com-

ponents that are waste products of the meat industry. (e.g., gelatin; bovine cornea). Level 5: *in vitro* with test components that have previously been exposed to animal product (e.g., human cell lines originally derived in FBS; some human tissue models). Level 6: *in vitro* animal-product-free with test components derived ethically from humans. Level 7: *in vitro* animal-product-free, fully defined. Any cell culture method using FBS is classified as Level 2 – only one level up from animal testing – due to the suffering involved. While Level 7 tests are desirable both scientifically and ethically, this is not always possible using currently available culture systems. However, even small changes, such as a switch from FBS to human serum, can elevate a test from Level 2 to Level 5/6. We will look at examples where this approach has been used to adapt skin sensitisation tests accepted at OECD level.

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**Presentation:** Oral

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## Implementing transparency in Portugal

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The Portuguese Transparency Agreement was initiative initiated by the Portuguese scientific community in collaboration with the European Animal Research Association (EARA) to promote a more consistent approach to communicating the scientific, ethical and moral justifications for animal research in Portugal. The signatories have committed to providing open and transparent information about the research involving animals and the standards of animal care and welfare carried out in the institution. The signatories only use animal models in research when there are not validated alternative methods.

Signing the Portuguese Transparency Agreement has given the organisations the opportunity of building a collective institutional approach to improving openness and transparency with the public, has helped to commit the researchers to be prepared to provide information to the media and the general public on the conditions under which animal research is carried out and how the results obtained, has brought scientists to build up an engagement in a dialogue with the society to improve the level of understanding the reasons why animals are still needed for biomedical research. The next steps will be presented and discussed.

**Presentation:** Oral



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## Inclusive culture of care at a global CRO: A necessary adjunct to governance

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It has long been known that a robust governance and a rich culture of care are two necessary pillars supporting the delivery of high standards of care by health care organizations. Whilst a strong governance framework provides the means to ensure that appropriate standards are adequately embedded, timely implemented and regularly monitored, a rich culture supports high quality health care by enabling, motivating and empowering employees to express their compassionate feelings and to take appropriate decisions in a timely manner when facing unexpected situations. As a global preclinical contract research organization conducting tests in animals to assess the safety of drugs, medical devices and chemicals, Covance works in a highly regulated (externally and internally) and process-driven environment including animal welfare, drug discovery and development, and good laboratory practices (GLP), regulations (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (<https://eur-lex.europa.eu/eli/dir/2010/63/2019-06-26>), Guidance on the Operations of the Animals (Scientific Procedures) Act 1986 ([https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment\\_data/file/662364/Guidance\\_on\\_the\\_Operation\\_of\\_ASPA.pdf](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/662364/Guidance_on_the_Operation_of_ASPA.pdf)), Animal Welfare Act and Animal Welfare Regulations ([https://www.aphis.usda.gov/animal\\_welfare/downloads/AC\\_BlueBook\\_AWA\\_508\\_comp\\_version.pdf](https://www.aphis.usda.gov/animal_welfare/downloads/AC_BlueBook_AWA_508_comp_version.pdf)), Guide for the Care and Use of Laboratory Animals (<https://olaw.nih.gov/sites/default/files/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf>) and guidance. In 2019, Covance reached out to more than 5,000 employees to renew our commitments to animal welfare using a modern approach based on inclusiveness and designed to facilitate the expression of emotional bonding and compassion. The presentation will review strategies to prepare, implement and continuously nurture a culture of care globally in a complex setting. It will also discuss how an all-inclusive and actively supported culture of care complement governance mechanisms in their common goal of ensuring responsible research characterized by respect for animals and focus on the 3Rs, data quality and regulatory compliance.

**Presentation:** Oral

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## Introduction of KSAAE

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The aim of Korean Society for Alternative to Animal Experiments (KSAAE) is to invigorate research on developing new test methods that would minimize the suffering of experimental animals and reduce the number of animals used, and ultimately replacing the use of laboratory animals in connection with animal experiments that are inevitably performed for human health, welfare, and academic development. Also, the aim of KSAAE would include the promotion of the public understanding through the exchange and education of related scientific information.

The foundation of the KSAAE is originated from the Research Association for Alternative to Animal Experiments that was established in July 2006, which later became KSAAE. The founding general meeting and the formal launch of KSAAE took place in National Institute of Toxicological Research of Korea in February 2007.

In addition to the establishment of the KSAAE, the Journal of Alternative to Animal Experiments, an official journal of KSAAE, was launched to publish the results of research related with alternative to animal experiments.

Since its inception, the KSAAE has held international conferences once or twice a year and has continuously published the official journal to encourage the study of alternative to animal experiments in Korea.

In March 2013, the European Union has banned the sales of animal tested cosmetics, and in December 2013, the Regulation on the Evaluation of Functional Cosmetics in Korea was amended to apply the alternative test to animal experiments approved by the OECD or the Korean FDA. Recently, there is an increasing demand for development of alternative to animal experiments in academia, industry, and government sides.

In line with the recent changes in concept for animal experiments to alternative studies worldwide, it is expected that the studies on alternatives to animal experiments will be more actively conducted, and the KSAAE will continuously support these efforts.

**Presentation:** Oral



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## Good Cell Culture Practice for stem cells and stem-cell-derived models

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The first guidance on Good Cell Culture Practice (GCCP) dates back to 2005 (Coecke et al., 2005). With the availability of human induced pluripotent (2006) stem cells, the potential applications of human cell culture models have been greatly broadened. When using stem cells and stem-cell-derived models in research, product development, testing and manufacture of biotechnology products and cell-based medicines, it remains critical to include aspects of quality assurance. As such, the original set of GCCP principles of best practice can be used as a basis to assure good cell and tissue practices and conditions when working with stem cell derived test systems in simple set-ups or in very technological advanced formats (Pamies et al., 2017). GCCP and its next generation principles (GCCP 2.0) are intended as guidance to obtain reliable and relevant study data. Applying GCCP as part of overall Good In Vitro Method Practices (GIVIMP; OECD, 2018) by the global life science community is leading to more harmonization of *in vitro* method related processes and procedures. Researchers are key players to ensure use of such best scientific and quality practices and are ideal ambassadors to use them in the novel generation of stem cell and tissue-based methods. The Eu-

ropean Commission Joint Research Centre's European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) is working on developing and validating innovative, mechanistic methods and approaches based on current harmonized scientific and quality standards complying with the GCCP principles and the GIVIMP guidance document (OECD, 2018) and is crowdsourcing for introducing stem cells and stem-cell-derived methods into the required regulatory test batteries. Key instruments to disseminate globally harmonized good cell, tissue and method practices are published principles and guidance, conferences, face-to-face meetings, training, webinars, templates (Krebs et al., 2019), reporting and evaluation tools (e.g., <http://www.scirap.org/>) and e-learning training materials.

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**Presentation:** Oral

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## Identifying optimized decision criteria and experimental designs by simulating preclinical experiments *in silico*

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Low statistical power in preclinical experiments has been repeatedly pointed out as a roadblock to successful replication and translation. To increase reproducibility of preclinical experiments under ethical and budget constraints, it is necessary



to devise strategies that improve the efficiency of confirmatory studies.

To this end, we simulate two preclinical research trajectories from the exploratory stage to the results of a within-lab replication study based on empirical pre-study odds. In a first step, a decision is made based on exploratory data whether to continue to a replication. One trajectory (T1) employs the conventional significance threshold for this decision. The second trajectory (T2) uses a more lenient threshold based on an *a priori* determined smallest effect size of interest (SESOI). The sample size of a potential replication study is calculated via a standard power analysis using the initial exploratory effect size (T1) or using a SESOI (T2). The two trajectories are compared regarding the number of experiments proceeding to replication, number of animals tested, and positive predictive value (PPV).

Our simulations show that under the conventional significance threshold, only 32 percent of the initial exploratory experiments progress to the replication stage. Using the decision criterion based on a SESOI, 65 percent of initial studies proceed to replication. T1 results in the lowest number of animals needed for replication ( $n = 7$  per group) but yields a PPV that is below pre-study odds. T2 increases PPV above pre-study odds while keeping sample size at a reasonably low number ( $n = 23$  per group).

Our results reveal that current practice, represented by T1, impedes efforts to replicate preclinical experiments. Optimizing decision criteria and experimental design by employing easily applicable variations as shown in T2 keeps tested animal numbers low while generating more robust preclinical evidence that may ultimately benefit translation.

**Presentation:** Oral

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## IACUC function and membership training in Korea: The first twelve years

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The Institutional Animal Care and Use Committee (IACUC) is responsible for oversight of animal care and use program and its components, as described in the Korean Animal Protection Act Article 26 (IACUC Function). The Animal Protection Act

that regulates animal use for scientific purposes reflects the 3Rs principles and ethical review process. The Act was introduced in 2008 and has been revised a total of 27 times. The Minister of Agriculture, Food and Rural Affairs supervises IACUCs and has announced the 3<sup>rd</sup> five-year (2020 to 2024) master plan to promote animal welfare and IACUC function. One of six main themes in the plan strengthens the ethical review policies for laboratory animal use, for the first time as a focused theme that is properly budgeted, in contrast with the previous two five-year plans that began in 2010. The framework for IACUC membership appointments was revised in 2013 and 2017, with a minimum of four hours of mandatory education is required beforehand. The Commissioner of the Animal and Plant Quarantine Agency is responsible for providing course guidelines (Notice 2017-33) and for either conducting a course directly or designating a course organizer. The Bioethics Information Centered (BIC) Study was approved as a course organizer in October 2018, and this status has been renewed every year since then. The first two authors have published an article reviewing course curricula, based on the legal requirements and types of training programs conducted by two Korean government regulatory agencies: the Animal and Plant Quarantine Agency and the Ministry of Food and Drug Safety. By the end of 2019, a total of 410 IACUCs and 2,451 IACUC members had registered and a total of 3,194 IACUC members had been trained since 2008. This paper will summarize the progress and challenges associated with these training experiences.

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**Presentation:** Oral



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## Round table: Industry and public sector partnerships in education to foster the implementation of alternative methods to animals in education and risk assessment: An overview with special reference to Indian context

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In India “alternatives” is not a priority. The regulatory authorities, other than CPCSEA, enact laws but do little to implement the change among academia and industry by way of motivation, facilitation and training. Therefore, Mahatma Gandhi-Doerenkamp Centre (MGDC), established at Bharathidasan University, Tiruchirappalli, by Doerenkamp-Zbinden Foundation, in a tenured grant, took over this responsibility. MGDC was then taken over by UGC as National Centre for Alternatives to Animal Experiments (NCAAE). Society for Alternatives to Animal Experiments-India (SCAAE-I) has also been established. The chronology is: MGDC (2009-2016)→NCAAE (2016-2021)→SCAAE-I (2018→). PeTA-India, PfA and HSI-I push the academic endeavors forward. The Center/Society liaises with open-minded industry partners in its endeavor. Thus, MGDC went into alliance with Episkin Academy, France; and In Vitro Admet Labs, USA, and conducts training workshops, one or more each year, at designated institutes/labs across the huge country. The trainers are doctoral students, researchers and technical personnel in CROs. The 3-4-day workshops concern *in vitro* toxicology in 2D and 3D formats. Episkin Academy provides training in use of reconstructed human epidermis in risk assessment of cosmetics/ingredients and/or medical devices. The In Vitro Admet Lab imparts training in integrated discrete multiple organ co-culture (IdMOC). The approach includes lectures and presentations on the principles and practices of IVT and the respective domains, and then takes on practical training to the end result and analysis. The philanthropy is par excellent- the alliance partners do not levy any charge. The cosmetic industry and academic community are greatly benefitted by the Episkin Academy training. Academic and research communities are benefitted by IdMOC training. Episkin Academy further awards prizes and travel grants to and through the Society. The University-Public Sector partnerships, thus, are models to emulate. More alliances are invited such as to empower the stakeholders and obviate animal experiments.

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**Presentation:** Oral

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## Transcriptome guided approaches to mimic homeostatic adult microglia in culture

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Microglia, the tissue macrophages of the central nervous system (CNS), are the principal resident cells that drive inflammatory responses. They modulate both innate and adaptive immune responses that occur in the CNS and are key players during brain homeostasis as well as during neurodevelopmental and neurodegenerative disorders. Detailed knowledge of their cell biology is therefore of pivotal importance, but it has proved difficult to set up reliable *in vitro* methods.

Recent studies have demonstrated that even the RNA transcriptomes of primary microglia *in vitro* cultures only partially recapitulate that of *in vivo* microglia. With the aim to characterize bottlenecks and master regulators, we have exposed primary microglia from adult rhesus macaques to a variety of different culture conditions, and compared their transcriptomes to those of mature, homeostatic *in vivo* microglia. Regardless of culture conditions, we found major differences in the gene expression profiles of *in vitro* and *in vivo* microglia. which is in line with recent data from other labs. Analysis of the differentially expressed genes enabled us to develop a new, partially serum-free, mono-



culture protocol for primary adult microglia, that yields high numbers of cells with a complex ramified morphology. Further analysis marked the importance of CNS-specific cues and the extracellular matrix. Based on these data we are now pioneering different 2D and 3D mono- and coculture systems, of which results will be presented. Our ultimate aim is to add the natural resident inflammatory system to the *in vitro* toolbox to study CNS disorders. The optimization of *in vitro* microglia cultures is important to facilitate *in vitro-in vivo* translations and contributes to the replacement and reduction of animal research.

**Presentation:** Oral

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## Publication bias: The problem that needs to go away

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Publication bias: the problem that won't go away. That's the title of a 1993 study by Kay Dickersin, then at the Department of Epidemiology and Preventive Medicine at the University of Maryland, and Yuan-I Min at the Johns Hopkins University. In their study, published in the *Annals of the New York Academy of Sciences*, the two authors define publication bias as "any tendency on the parts of investigators or editors to fail to publish study results on the basis of the direction or strength of the study findings." Currently, publication bias is recognized as the preference of journals for publishing studies with positive results while rejecting those with negative ones. However, in February 2013, Dr. H. Shaw Warren, then a sepsis researcher at Massachusetts General Hospital, reported difficulties in publishing his research group's study because reviewers did not accept his study's main finding: mice are very poor models for mimicking human inflammatory diseases. Similar situation happened to a paper published by Dr. Hans Clevers' group (Utrecht University) in 2019 and, as reported by Dr. Donald Ingber (Harvard University), his research group has also encountered similar situation, many times. Episodes like these define a recently recognized type of publication bias in which editors and/or peer reviewers of specialized journals request animal data to validate studies produced using human biology-based approaches, such as human organoids. What are the roots of this type of publication bias? Some possible explanations are a lack of understanding of how advanced alternatives work, resistance to the new, conflict of interest, journal editorial policy, and sunk costs (tendency to continue an endeavor once an investment in money, effort or time has been made). In this round table we will discuss strategies to prevent this type of publication bias from happening.

**Presentation:** Oral

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## Editors' moral obligations – Profit, regulation and virtue

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Ethics is the branch of knowledge or study dealing with moral principles. These principles guide actions and choices made by individuals. A moral obligation is a constraint on action and choice that may arise from legal or ethical considerations. Underlying principles that impose obligations on journal editors arise from considerations of profit, regulation and virtue.

To profit is to gain. Why might a journal exist? What do those responsible, e.g., learned societies and publishers, hope to gain from publishing a journal? What is their interest: financial reward, prestige, impact, social good?

Journals and editors function within regulatory frameworks that may be internally or externally imposed. Most journals have "guidelines for authors", which may be extensive and detailed or relatively light-touch. External frameworks include the Committee on Publication Ethics (COPE), founded in 1997. There are many other guidelines of relevance to journal editors that address specific issues: ARRIVE, CASP, FAIR, PREPARE, PRISMA, TOP to name but a few. Where do these regulations come from? Why are there so many and do they make journals better?

Dr Ed Pellegrino argued that any professional (doctor, lawyer, scientist, editor) should be both competent and virtuous. Such an editor embodies habits and instincts that inform choices that promote fundamental goods, irrespective of regulations or interests. A competent and virtuous editor does not require regulations to know that published science should be conducted ethically and be of a good quality.

These three dimensions of moral obligation must function harmoniously and not in competition. Clarity as to whether "Open Science" and "Transparency" are interests, regulations or fundamental goods is essential and perhaps not so obvious. To act effectively and ethically, an editor must have clearly defined objectives and follow a transparent regulatory framework. However, these must be built on a secure foundation of personal and institutional virtue.

**Presentation:** Oral

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## Human liver-pancreas-heart microphysiological system for studying cardio-metabolic disorders

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The standard *in vitro* tools using single cell types or single organoids are not capable of replicating complex disease progress or homeostatic crosstalk of two or more organs, hampering the study of metabolic disorders. To address this challenge, we developed a human three-organ microphysiological system (MPS) including liver, pancreas and heart. We modified and extended our liver-pancreas model (Bauer et al., 2017) by replacing the HepaRG cell line with primary human hepatocytes and by including human cardiac spheroids as a third organ to study cardio-metabolic diseases.

Liver spheroids were formed of primary human hepatocytes and human hepatic stellate cells, pancreatic islets were purchased from InSphero, and cardiac spheroids consisted of human induced pluripotent stem cell derived cardiomyocytes and primary human cardiac fibroblasts. We coupled the microtissues using a multi-compartment chip platform with pulsatile flow of culture medium (Wagner et al., 2013). We equipped each tissue compartment with a porous microcavity membrane to maintain proper three-dimensional architecture of spheroids and followed viability and organ-specific functions over time. For analysis of cardiac spheroids, we optimized assay conditions for the live-cell metabolic assay platform (Seahorse) to evaluate energy metabolism and used automated time-lapse imaging and a mathematical algorithm to measure beating rate.

In a proof-of-principle three-organ study, all three microtissues remained viable over two weeks of culture as measured by lactate dehydrogenase release. We showed that organ-specific functions such as albumin and urea production of liver spheroids, glucose-stimulated insulin secretion of islets, and beating rate of cardiac spheroids were stable over time.

Multi-organ models are key to improve studies on systemic disease progression that involves crosstalk of several organs. Our liver-pancreas-heart MPS is a promising tool to simulate complex cardio-metabolic disorders *in vitro* and could potentially be used to investigate new drug target and therapies.

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**Presentation:** Oral

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## The development of alternative methods in China and the role of the industries

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The outspread of alternative methods to animal testing needs industries play an indispensable role. Under the situation of the top-down banned the animal testing with great uncertainty, the Consensus Center of Alternative Research and Evaluation Method (CCARE), jointly established by department agency, educational institutions and the cosmetics industries, promoted alternatives development in China during the past 12 years. CCARE activities have covered education, training, academic exchanges and scientific popularization. Taking the alternative methods based on the skin model as an example, in 2011, the me-too validation of OECD439 based on EPISKIN produced in Shanghai was carried out by 5 multi-industry laboratories, which is only the validation activities completely in accordance with the OECD guideline still up to now. After validation, the inspection and quarantine industry standards were formed, which now is used by many companies. At present, joint third-party inspection agencies and industries, the efficacy methods based on the full thick skin model T-SKIN has been developed, which can be used for research and testing for anti-photoaging, anti-oxidation, anti-PM2.5 pollution, ozone, skin lesion repair, fungal infection. The organ-on-a-chip platform which established by Chalet Co., has innovated a skin-on-chip system combine skin equivalent with hair follicle, sensory neuron, dendritic cell and adipocyte, which enrich the efficacy assessment tools. With the support of industries including *in vitro* system suppliers, third party laboratories and manufacturers, the consensus and development will be accelerated in the near future. (Fund by Guangzhou ETDZ International Scientific and Technological Cooperation Project, 2017GH11)

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**Presentation:** Oral



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## Cartilage-on-chip: Towards improved models of osteoarthritic disease

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Osteoarthritis is an invalidating disease characterized by gradual articular cartilage deterioration affecting 1.4 million Dutch and costing 18 billion-€ in 2018. The lack of translation power of current *in-vivo* and *in-vitro* models contributes to the estimated increase in treatment costs to 54 billion-€ in 2040. Consequently, the developed predictive model that accurately emulates the tissue's microphysiology (e.g., organ-on-chip) is urgently needed. Articular cartilage is an avascularized structure comprising specialized cells (chondrocytes) experiencing both compression and shear stress generated through the sliding of the two bones. Our platform mimics the 3D structure of the tissue and incorporates multi-axial mechanical stimuli, as present in the joint (Paggi et al., 2020), emulating shear strain and physiological/hyper-physiological compression.

The platform, in polydimethylsiloxane, comprise three sections: a mechanical actuation chamber; a cell-hydrogel section; and a perfusion section, to provide nutrients. Human chondrocytes (hCHs) were cultured in agarose hydrogel within the platform using differentiation medium. The cell projected area deformation was determined by applying 7 individual pressures from the mechanical actuation system. Cell viability was evaluated for different mechanical stimulation conditions. Glycosaminoglycan production was assessed using Alcian blue and nuclear fast red on histology sections after 15 days of culture in the platform.

Cell area decrease is correlated to the cell location in the device with respect to the membrane and the applied pressure. The hCHs cultured with hyper-physiological compression (> 20%) showed a significant decrease in cell viability (day 3), while under physiological conditions (< 20%) they maintained their viability after 15 days of stimulation. hCHs were next cultured in static or dynamic conditions (compression or combination of compression and shear strain). Here, Glycosaminoglycan production demonstrated that a combination of compression and shear strain greatly enhance matrix formation.

This platform allows mimicking of healthy and hyper-physiological stimulation, which is instrumental in studying disease progression and drug uptake and response.

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**Presentation:** Oral

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## Identification of endocrine disrupting chemicals in fish embryos

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Embryonic stages of fish are considered to feel no or less pain or distress. Therefore, European welfare regulations classify fish embryos as non-protected life stages. Hence, they are considered as alternative testing method. They share the small scale and high-throughput capacity with cellular *in vitro* models. In contrast to genuine *in vitro* methods, however, they reflect the complexity of an entire organism. This is of particular importance for identification of endocrine disrupting chemicals (EDCs), which may not only interact directly with hormone receptors but also interfere with hormone synthesis and feedback loops of hormonal regulations. The latter would be difficult to address in cellular *in vitro* methods. Furthermore, endocrine disruption in fish embryos can be studied in the context of other modes of action beyond cytotoxicity, e.g., (neuro)developmental toxicity. Typically, non-EDC effects can be tested within the same test setup for endocrine disruption. This enables to estimate the relevance of endocrine disruption with respect to other effects or to indicate if observed endocrine effects may relate to secondary unspecific responses. In the presentation the different available methods for assessment of endocrine disruption in fish embryos and the pros and cons are discussed. As an example, the analysis of thyroid hormone disrupting chemicals in zebrafish using the transgenic strain *tg:mcherry* is demonstrated. Disruption of hormone synthesis leads to the activation of the feedback loop for increased expression of thyroglobulin. Due to a construct of the thyroglobulin promoter and the red fluorescent protein *mcherry* induction of thyroglobulin is monitored by an increased red fluorescence in the thyroid gland. By automated positioning of fish embryo and fluorescence microscopy, the thyroid gland fluorescence is quantified and used to establish concentration response curves (Jarque et al., 2019). Furthermore, an approach for the parallel and automated assessment of (neuro)developmental toxicity is demonstrated (Teixido et al., 2019).

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**Presentation:** Oral



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## From case studies to a regulatory guidance: The EU-ToxRisk NAM-assisted RAX advisory document

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Grouping/category approaches for read-across have evolved over the last decades as important risk assessment tools in order to attempt filling data gaps in particular for complex endpoints such as repeated dose or developmental and reproductive toxicity without performing additional animal studies (ECHA 2014). To date many read-across cases fail to demonstrate toxicokinetic and toxicodynamic similarities. New concepts involving an integrated application of *in vitro* and *in silico* tools are needed to better characterize common properties of structural similar chemicals, collectively called New Approach Methodologies (NAMs). EU-ToxRisk has developed a NAM-based read-across strategy (Escher et al., 2019) and systematically evaluated this strategy in several experimental case studies addressing repeat dose toxicity and developmental and reproductive toxicity. Case studies involved both *in silico* and *in vitro* animal-free approaches targeting both toxicokinetics and toxicodynamics aspects. The case studies have been systematically reported using read-across reporting templates from ECHA and OECD, and subsequently been evaluated by an international regulatory authority panel under guidance of the OECD. These case studies have resulted in important learnings which led to an EU-ToxRisk read-across regulatory advisory document. These learnings will be presented in this presentation. Altogether our work strongly encourages the toxicological community to integrate a mechanism-based risk assessment and its regulatory acceptance for read-across assessment.

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**Presentation:** Oral

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## VitalTissue, an initiative to supply viable human materials to laboratories in the Netherlands

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Drug safety and efficacy testing is frequently performed in laboratory animals, with limitations regarding extrapolation to humans. For the development of better translational methods, viable human tissue or cells are preferred over animals and immortalized cell lines, especially since several human (tissue specific) models have become available by recent scientific developments (e.g., models based on human tissue or iPSC derived cells/organoids). However, availability and access to high-quality viable human tissues is a hurdle for many researchers.

Here we present the initiative VitalTissue (<http://www.vitaltissue.nl>; <https://bit.ly/3rpuiP0>), a platform that aims to supply surgical residual tissues to researchers in end-user laboratories in a (financially) sustainable and transparent way. The initiators of the project have interviewed stakeholders (e.g., researchers, surgeons, patients) and conducted qualitative interviews and quantitative (online) polls and conclude that there is a substantial unmet need for various types of human tissue, and that potential donors would generally consent to the use of their left-over tissues after medical procedures. We have addressed the legal, ethical, biosafety and logistic issues involved in the supply chain, and developed protocols for the VitalTissue supply chain regarding criteria for sample specification (tissue and donor inclusion criteria), logistics, and information management. Using these protocols, pilot studies were performed in which residual tissue (skin, liver, gut) were supplied from hospitals to researchers within the consortium in order to evaluate the (metabolic) viability of the human tissue samples. Future research will focus on the development of a framework for the long-term qualification of suppliers and end users. Project governance includes the involvement of societal stakeholders and high-level public information on the development of the tissue supply chain. In conclusion, VitalTissue is a promising initiative for a supply chain of residual human tissue samples, which is widely supported by multiple organizations with multidisciplinary expertise.

**Presentation:** Oral



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## Pain assessment and management in bone-linked mouse models

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Stable fixated osteotomies lead to moderate strain for the animals, while impaired fracture healing can only be modelled by a combination with secondary diseases, procedures or instable fixation which might be accompanied by severe suffering of the animals. The selection of analgesics in bone healing models is restricted due to potential interfering properties of anti-inflammatory drugs. The application of analgesics via the drinking water might be a less stressful alternative to repeated injections. However, studies that address the efficiency of Tramadol, a widely used analgesic in bone-linked models, in the drinking water are scarce. Different recommendations exist regarding the dosage, and they widely differ from potentially under- to overdosing. In order to enhance the knowledge on refinement options and to effectively reduce lab animal usage, we performed a refinement study embedded in a basic research mouse osteotomy study. We evaluated two commonly used pain management protocols, Tramadol (two concentrations) and Buprenorphine in the drinking water, for their efficiency and side effects on experimental readout in a mouse osteotomy model. By monitoring (i) general parameters of wellbeing, e.g., clinical scoring, weight and (ii) model specific pain parameters, we reported that Tramadol at higher dosages does not provide extra benefit in pain reduction but negatively affects the wellbeing of the animals. Tramadol in the lower dosage or Buprenorphine seem to be sufficient in alleviating pain in this model. We are currently planning a follow-up study to further optimize our protocol with, e.g., intra-animal control for anesthesia effects, Tramadol application via the drinking water one day prior to surgery or the application of a sustained-release formulation of Buprenorphine. Our results gained in a “normal” mouse osteotomy model are of great importance to be transferred to animal models of impaired fracture healing in order to effectively apply refinement strategies and to reduce severe suffering.

**Presentation:** Oral

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## Advancing Three Rs education and training under a European Parliament Pilot Project at EURL ECVAM

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In accordance with Article 48 and Annex VII of Directive 2010/63/EU on the protection of animals used for scientific purposes, the JRC's EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) provides support to develop, validate and promote alternatives to animal procedures in the area of regulatory testing and in basic and applied research.

Recent EURL ECVAM studies have shown that although much Three Rs knowledge exists, its sharing could be improved especially between different fields of expertise through better coordination, communication and outreach, and by more emphasis on targeted education and training initiatives.

With this in mind and benefitting from funding made available under a European Parliament Pilot Project (2018-2020), which aimed to promote alternatives to animal testing including the application of the Three Rs, EURL ECVAM launched a project to investigate the feasibility of including the Three Rs in educational curricula. The project focused on high school, university and early career education levels and has produced the following: a guidance document that informs key education decision-makers on how to facilitate the incorporation of the Three Rs into their programs and curricula, specifications for educational resources that promote the Three Rs at the three levels of education and some practical resources that can be used at all levels. Under a sub-project with high school education specialists, lessons on the Three Rs have already been prepared and delivered to students under this pilot.

**Presentation:** Oral



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## High throughput transcriptomics to derive mode-of-action and potency information to support read-across approaches

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Chemical read-across is commonly evaluated without particular knowledge about the biological mechanisms leading to the observed adverse outcomes in *in vivo* studies. The integration of data showing a shared mode of action, preferably in human-based test systems, will strengthen the read-across assessment. EU-ToxRisk has applied high throughput transcriptomics approaches to provide mechanistic insight on similar mode-of-action as well as potency of structural similar chemicals and/or chemicals with a identical molecular initiation event. In this presentation we will highlight the findings of high throughput transcriptomics analysis of i) a panel of > 15 valproic acid (VPA) analogues that have various propensities to induce liver steatosis and ii) a panel > 10 mitochondrial pesticides, targeting complex 1, 2 or 3 of the respiratory chain and causing mitotoxicity. Primary human hepatocytes and the human HepG2 liver cell line were exposed to a concentration range of the chemicals and analyzed at 24 h. We used a TempO-Seq targeted high-throughput screening assay to determine the differential expression of > 3000 genes, reflecting all biological pathways. Systematic bioinformatics analysis of concentration response information of individual target genes and co-regulated gene networks allowed a systematic comparison of biological similarity and potency, as well as the identification of sensitive biomarkers to define liability for steatosis and mitotoxicity. The presentation will explain the transcriptomics strategy, highlight the results and share the implications and opportunities for chemical safety testing.

**Presentation:** Oral

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## Use of non-animal models for the hazard identification of endocrine disrupting properties: A regulatory perspective

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The hazard identification of pesticides having endocrine disruption properties is conducted according to the guidance published by EFSA and ECHA in 2018 (ECHA and EFSA, 2018). The guidance implements the Regulation 2017/2100 and 2018/605 for biocides and pesticides, respectively.

For investigating endocrine activity, the strategy in the ECHA/EFSA Guidance (2018) suggests first conducting level 2 tests (*in vitro* mechanistic assays) (OECD 150, 2018) in order to reduce animal testing and facilitate the Mode of Action (MoA) analysis. However, negative results *in vitro* are not considered sufficient to demonstrate lack of endocrine activity *in vivo* due to the limitations of the currently available *in vitro* test systems (lack of metabolic system, no consideration of ADME, use of mammalian cell lines, only, etc.).

For non-target organisms, the ECHA/EFSA Guidance (2018) considers endocrine activity sufficiently investigated, if an “Amphibian metamorphosis assay” (AMA; OECD 231, 2009) is available for the thyroid modality and a Fish-Short Term Reproduction Assays (FSTRA; OECD 229, 2012) for estrogen, androgen and steroidogenesis modalities.

Several embryonic assays, which are placed at level 3 of the OECD conceptual framework (OECD 150, 2018), are at different stage of development. Those assays utilize eleutheroembryos, thereby relying on a more complex model (including kinetics, metabolic competence, developed organs etc.) without the protection of the chorion. Furthermore, since these models are used up to feeding stage, they are excluded from the definition of a laboratory animal according to Directive 2010/63/EU and thus are compliant with the 3R principles. An OECD test guideline has been recently published for one of those, the *Xenopus* Eleutheroembryonic Thyroid Assay (XETA; OECD 248, 2019).

The aim of this work is to present an overview of the assessment strategy for the investigation of endocrine activity of the ECHA/EFSA Guidance focusing on the regulatory perspective regarding the inclusion of embryonic assays.

### References

ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the JRC (Joint Research Centre) et al. (2018). Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. *EFSA J* 16, e5311. doi:10.2903/j.efsa.2018.5311



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**Presentation:** Oral

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## New read-across modules for safer chemicals

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The reduction of *in vivo* tests to be conducted, have become indispensable in terms of resources and animal benefits. The read-across approach reduces the number of chemicals to be tested because the available information about the endpoints can be used to estimate those properties for untested substances. This study describes a general read-across assessment concept to support toxicity characterization of chemicals by applying the modules developed within the framework of in3 project. The applied approach started with the implementation of different methodologies to select the most suitable analogues, where components as: structural similarity, physical-chemical similarities, structural alerts endpoint specific, and metabolism similarities were considered. Interpretable and flexible workflows implemented in KNIME were used to identify analogues, illustrating how *in silico* and *in vitro* tools can be combined within a read-across assessment to strengthen the read-across hypothesis. The analysis was based on diverse strategies combining supervised and unsupervised methods. Mechanistic knowledge such as structural alerts was generated and utilized, and case studies were conducted.

**Presentation:** Oral

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## The use of non-animal models in regulatory evaluation of environmental endocrine disruptors – An industry perspective

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Regulation (EU) 2018/605 and 2017/2100 outlining criteria for the identification of endocrine disruptors (ED) currently apply to pesticides and biocides, respectively. These rely upon the WHO/IPCS definition, which requires the identification of a biologically plausible link between an endocrine mode of action and adverse effects that are, for environmental species, relevant at the population level. According to the agreed definition, demonstrating a lack of endocrine activity is sufficient to conclude that the ED criteria are not fulfilled. However, the tests recommended in the ECHA-EFSA ED Guidance (2018) to assess endocrine activity in aquatic vertebrates require a significant number of animals (cf. OECD TGs 229 and 231). To date industrial chemicals have been addressed on a case-by-case basis under the CoRAP procedure where additional data are requested based on specific concerns. However, discussions are on-going to amend the REACH information requirements for endocrine assessment. The situation is even more complicated for the environmental safety assessment of cosmetic and personal care products, where the testing on vertebrate animals is prohibited. To fulfill current and future regulatory requirements for environmental ED assessment, the chemical industry is engaged in scientific initiatives to support (i) the use of cross-species extrapolation based on the evolutionary conservation of endocrine pathways across vertebrate taxa (cf. the US-EPA software tool SeqAPASS), (ii) the development of *in silico* approaches (e.g., QSAR bioactivity models for ER and AR), (iii) the development and validation of omics approaches and *in vitro* models, (iv) the validation of non-animal eleutheroembryonic assays, and (v) the implementation of integrated mechanistic modeling approaches combining TK/TD and population models within the quantitative adverse outcome pathway framework. We are optimistic that these engagements with developing policy and scientific approaches will contribute to robust approaches to the assessment of ED for environmental species that employ fewer vertebrate animals.

### References

ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC) et al. (2018). Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. *EFSA J* 16, e5311. doi:10.2903/j.efsa.2018.5311



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**Presentation:** Oral

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## Advancing Three Rs education and training under a European Parliament Pilot Project

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In order to further promote the implementation of Directive 2010/63/EU, the European Commission issued calls for a number of related projects last year. One of these projects is aimed at facilitating the uptake of non-animal alternatives by developing two e-learning modules. The contract for this project was awarded to a consortium consisting of SYRCLE, the Swiss 3R Competence Centre, Institute for In Vitro Sciences, Pharma Launcher and Ecorys UK. This consortium will develop two modules, i.e., one e-learning module focused on searching for existing non-animal alternatives (including systematic reviews) and one module targeted at researchers who want to develop reliable and relevant non-animal alternatives for regulatory use taking into account Good In Vitro Method Practices (GIVIMP). The quality of the developed modules will be assessed by external review groups. The learning outcomes will be presented to the commission along with the design of the assignments through which these outcomes will be realized.

**Presentation:** Oral

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## Integration of *in silico* and *in vitro* NAM approaches to support read-across: The EU-ToxRisk experience

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There is a need for a paradigm shift in toxicology towards an animal-free, mechanism-based integrated approach to human chemical safety assessment involving both *in silico* and *in vitro* approaches that are referred to New Approach Methodologies (NAMs). The EU-ToxRisk project has embraced an integrated NAM-based chemical safety approach and united all relevant disciplines and stakeholders to establish pragmatic, solid read-across procedures incorporating mechanistic and toxicokinetic knowledge. NAM approaches did include computational modelling approaches, high throughput molecular initiation event characterization, apical endpoint evaluation in advanced test systems and high throughput transcriptomics. The toxicodynamics data was integrated with detailed toxicokinetics data. The EU-ToxRisk has systematically evaluated the application of these NAM tools in various case studies in the context of read-across. The EU-ToxRisk case studies demonstrate the feasibility for the application of a mechanism-based testing strategy for animal-free chemical safety assessment. The presentation will exemplify the NAM-based testing approach and the implications as well as limitations for future safety testing.

**Presentation:** Oral

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## Where are we now: Replacement of *in vivo* skin sensitisation assays

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There are a number of methods available for the evaluation of skin sensitisation potential. While historically *in vivo* assays have been the established choice, we have seen the emergence of non-animal methods and approaches. The development of animal-free techniques, their ready adoption, and a concomitant reduction in the use of *in vivo* tests are essential in the drive to end all *in vivo* testing – in this presentation we will take a look at how far along we are in this journey and the hurdles yet to be overcome. The OECD is instrumental to the progression and global harmonisation of test methods. We will delve into their



test guidelines programme for skin sensitisation, look at the importance of updating guidelines and ponder: when is it the right time to delete or narrow the application of an *in vivo* method and could we be close to saying “bye-bye Buehler”? Cruelty Free International has expert stakeholder status at the OECD (as an active member ICAPO, the International Council on Animal Protection on OECD Programmes), and also at the European Commission, ECHA and at the European Centre for the Validation of Alternative Methods (as leader of Cruelty Free Europe).

**Presentation:** Oral

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## REACH and the 3Rs – Reinvigorating efforts towards the avoidance of *in vivo* testing

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The REACH regulation features mechanisms for the avoidance of testing on animals, but do they work? We will explore what these mechanisms are and evaluate their effectiveness. Although there has been some improvement to ECHA’s procedures following pivotal board of appeal cases, we argue that there has been little meaningful progress towards the ultimate Replacement goal of Directive 2010/63/EU. We will outline our view on the fundamental reasons for this and how the progression towards full replacement of all *in vivo* tests can be reinvigorated. As leader of Cruelty Free Europe, we have expert stakeholder status at the European Commission, ECHA and at the European Centre for the Validation of Alternative Methods. We also are expert stakeholders at the OECD (as an active member ICAPO, the International Council on Animal Protection on OECD Programmes). As an animal protection organisation with privileged access to regulatory activities across Europe, we very much look forward to sharing and discussing our insights with you.

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**Presentation:** Oral

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## Auditing for GLP compliance, a regulatory study that relies heavily on computerized prediction models and complex *in vitro* techniques, where are the pitfalls and issues to care about

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GLP and complex detection systems, test items and test systems: a current reality? New and complex test items, modern and innovative new technologies used in methods proposed for OECD test guidelines are challenging not only the scientific community, but also raise regulatory compliance issues. In the rapid evolving world, regulatory agencies are confronted with cloud-based applications, artificial intelligence with machine-learning process, block-chain, very complex test systems, test items, analytical equipment, pure *in silico* method (Q-SAR, predictive models) ...

For the regulatory safety evaluation of chemicals (and other new complex test items), there is a regulatory compliance requirement for these methods to be technically validated (accurate, robust and reliable) to ensure the generation of scientific relevant data. On the other hand, by respecting the GLP Principles, the test facility remains in full control of the integrity and the quality of the data.

With the implementation of complex methods, it has proven very difficult for end users (test facilities) to fully comply with the regulations when these new technologies have to be implemented, as sometimes crucial steps are out of their control.

To that end, it is of very high importance that continuous dialogue and collaboration between industry and regulators could be established. In order for regulators to be able to adapt and create new guidance on time, it is also important that the collaboration between all stakeholders starts as early as possible.

The final goal of this approach is to ensure the integrity and the quality of non-clinical safety data in compliance with the GLP regulation and the scientific relevance of them, so that the OECD Mutual Acceptance of Data agreement could still provide all the trust needed and continue to be a reality where all stakeholders and regulators can rely on.

**Presentation:** Oral



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## Predictive value of PBK-model predictions based on *in vitro* and *in silico* input data as essential tool in next generation (animal-free) risk evaluations

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A critical challenge facing next generation (animal-free) testing strategies for chemical safety evaluations is the conversion of *in vitro* toxicity data into *in vivo* dose-response or potency information. Physiologically based pharmacokinetic (PBK) modelling plays a crucial role in performing such quantitative *in vitro-in vivo* extrapolations (QIVIVE). To facilitate the application of PBK models in next generation risk evaluations, it is important to gain confidence in the predictive value of these models when developed solely based on *in vitro* or *in silico* input parameters. In this study different *in vitro* and *in silico* input strategies for PBK-model development were evaluated with respect to their performance to predict peak plasma concentrations (C<sub>max</sub>) in humans. C<sub>max</sub> predictions were made for 562 studies (i.e., exposure situations described in the literature with reported C<sub>max</sub> values) for 43 different compounds. Up to five orders of magnitude differences between predicted and observed C<sub>max</sub> ratios were observed for certain compounds, depending on the input approach that was applied. Differences between the applied calculation methods for partition coefficients contributed most to this wide range in C<sub>max</sub> outcomes. Different chemical characteristics were explored to explain deviations between the predicted and observed C<sub>max</sub> values. Overall, the results obtained can be used to define strategies to reduce uncertainties in internal dosage predictions based on *in vitro*- and *in silico*-based PBK models and to define the applicability domain of the models.

**Presentation:** Oral

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## Translating animal model results to human disease

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Animal models are extensively being used in virology, but the translatability of results from animal studies to humans is unknown. In this study, we aim to systematically review the translation of poliovirus infection of humans, causing poliomyelitis, as this virus has been widely studied with the use of animal models. We investigated, for the first time, how poliovirus infection in animals compares to humans, taking into account the route of infection, cell entry, replication sites and neurovirulence. We included 34 articles on animal studies and 26 reviews on human poliovirus pathogenesis following the search of the Medline and Embase databases. Quality was assessed using the SYRCLE's risk of bias tool for animal studies. We found poorly designed and conducted experiments: animal baseline characteristics were diverse, animal allocation and housing were never blinded or randomized. Overall, a high risk of selection, performance, and detection bias was observed. 27 different types of animal models were used, and results of animal and human studies were incongruent. We hypothesize that the lack of standardization of polio animal studies has led to the use of a wide variety of animal models which makes studies using these models difficult to compare. Variation between animal models may further have resulted in a poor translation of animal-model results to human disease. This poor animal model standardization could reflect the poor predictive value of animal model results for human virus disease.

**Presentation:** Oral

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## Iron fist & 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, re-



duce and refine their use of animals using both the velvety glove approach (education, support, encouragement) and the iron fist approach (regulatory action).

**Presentation:** Oral

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## Animal welfare bodies: Initial training and continuing professional development

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Directive 2010/63/EU requires Animal Welfare Body (AWB) to be set up by breeders, supplier and users (Art. 26). The AWB shall include at least the person or persons responsible for the welfare and care of the animals and, in the case of a user, a scientific member. In Art 23 (2) it is laid down that all staff shall be adequately educated and trained. Guidelines regarding such education and training have been developed and a modular training was suggested (1). While training of staff mentioned in Art. 23-26, as well as that of project evaluators, has been considered within the document, specific training needs for AWB personnel have not been addressed. As members of the AWB will most likely belong to one of the functions mentioned in Art 23 (2) of Directive 2010/63/EU, initial training could be covered by any of the core or function specific modules mentioned in the guidance document.

Today, no specific training programs have been established. Setting up (new) training schemes is time consuming and requires additional resources. Online trainings can help close the gap to facilitate training and CPD. They fit into nowadays complex work schedules and can be adapted to different educational needs for people from varied backgrounds within one platform. In this context, the e-Learning platform LAS interactive offers modular and customizable online training as well as a free information portal. Content can be extended through collaborations. Another project, 3R-SMART, is currently being developed to serve as an education and training platform, focusing on a collaborative approach as well. As part of the LAS interactive and 3R-SMART projects, we are currently working on developing a forum platform to assist collaboration and networking in laboratory animal science.

### Reference

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**Presentation:** Oral

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## Establishment of a human multi-organ-chip platform to replace animal transplant models for preclinical evaluation of Treg cell therapies

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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 regulatory T cell (Treg) therapy approaches for the prevention of transplant rejection are under evaluation. Adoptive transfer of Tregs is a therapeutic option to reshape intra-tissue immune imbalance in transplant patients. It aims at supporting long-term function of allografts by overcoming the challenge of undesired immune reaction by the recipient.

Here, we used the HUMIMIC<sup>®</sup> multi-organ-chip platform to establish a next-generation human *in vitro* assay for predictive preclinical testing of Treg products. The platform enables co-culture of various human 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<sup>®</sup> platform for two-organ co-culture exploring 3D printing tools and endothelial self-assembly processes. The organ models and endothelial cells were generated from iPSCs of two different individual HLA-tested healthy persons emulating the recipient and the donor background. Finally, we aimed to qualify a HUMIMIC<sup>®</sup> based next-generation transplant rejection assay to evaluate both, safety and efficacy of Treg products in a universal repeated dose long-term assay environment. Multi-organ-chip design and prototyping results are presented along with the results of iPSC-based differentiation of human endothelial cells, liver equivalents and kidney models for the establishment of the interconnected two-organ model. Furthermore, we present data on on-chip micro-vessel formation and co-culture over prolonged culture periods. Results will be discussed in the light of the assay potential to replace respective animal transplant models in use.

**Presentation:** Oral

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## Advancing machine learning and artificial intelligence techniques for use in (semi-) automatic literature reviews

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Use of machine learning and artificial intelligence techniques is spreading into a wide range of applications. Increasing speed of growth of the volume of published literature (peer-reviewed as well as grey) calls for efficient methods of processing large volumes of texts and data to allow fact-based decision making by policy makers, researchers and other stakeholders. Team of experts in literature reviews will present how artificial intelligence and machine learning can help in processing large volumes of data in systematic reviews. Case studies will be presented showing efficiency of various algorithms especially for screening of relevance of scientific literature to particular review questions and for subsequent (semi-)automatic extraction of key data.

Presented case studies will include the use of neural networks, support vector machines and other natural language processing algorithms for text clustering and named entity recognition in various scientific domains. In particular, comparison of performance of the developed algorithms will be presented based on recently carried out systematic reviews where the retrieved literature was annotated by experts in toxicology, food safety, molecular genetics and other scientific domains.

The presentation and the case studies will provide an overview of the state-of-the-art methods showing how artificial intelligence can be used to minimize the workload of expert reviewers, minimize the risk of errors in screening for relevance and extraction of relevant data, while also discussing current limitations and issues associated with further development of fully automated systematic reviews in different scientific fields.

**Presentation:** Oral

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## 3Rs leadership for young researchers

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Growing social and political pressure for development and implementation of the 3Rs will likely result in greater public and private investment, and therefore increase the number of opportunities for young researchers to obtain funding for, innovate on,

and lead the future of replacement, reduction and refinement. However, what background and profile young researchers should have to benefit from a (hopefully) increasingly favorable environment for the 3Rs is uncertain.

The case can be made that the 3Rs do not constitute a scientific field “*per se*”, but rather aggregate and benefit from progress in different scientific fields that – either specifically for that purpose or as a secondary outcome – can be applied to replace, reduce, or refine animal experiments. In this regard, young scientists working on a wide range of disciplines can focus or redirect their scientific activity to explore 3Rs-related challenges and opportunities. To be even aware of those, however, may require a level of understanding of past and current context of the 3Rs, as well as their ethical, scientific, and technical complexities, which is not part of the average scientist’s set of knowledge and skills, at any career stage, and may thus be far from their expertise or professional interest. Thus, it could be argued that there is also place for “*de facto*” 3Rs scientists, likely more generalists than specialists, that can be drivers for 3Rs innovation, by identifying emerging ethical and scientific challenges in animal research, bridging the gap between disciplines, and assembling and leading multidisciplinary consortia to address them.

This talk by a “Xennial” (between a “Gen Xer” and a Millennial) somewhere between junior and senior scientist, will discuss possible approaches for young scientists to apply transferable skills and seize – or create – opportunities in order to become players, recognized professionals, and leaders in the 3Rs.

**Presentation:** Oral

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## STopTox: An *in-silico* alternative to animal testing for acute systemic and topical toxicity

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Acute toxicity tests are used to identify chemical hazards that cause toxic effects following very short exposure times. Since 2009, animal testing for cosmetic products has been prohibited in Europe, and in 2016, US EPA published a guideline for waiving the so-called “6-pack” battery tests (acute oral toxicity, acute dermal toxicity, acute inhalation toxicity, skin irritation and corrosion, eye irritation and corrosion, and skin sensitization) to



reduce animal testing of pesticides. The ultimate objective of our project is to develop STopTox (Systemic and Topical Toxicity) software as reliable *in silico* alternative to current *in vivo* “6-pack” toxicity testing approaches. We have compiled, curated, and integrated the largest publicly available dataset for these 6-pack endpoints. These data were used to develop an ensemble of QSAR models that were validated in compliance with the OECD principles. The compiled and curated STopTox dataset includes 11,941 compounds with activity measurements for at least one of “6-pack” endpoints. We have shown that STopTox models afford higher accuracy of predicting human outcomes than animal testing. Virtual screening of the REACH database using the developed QSAR models identified multiple potential toxicants. STopTox models can be employed to identify both putative toxicants and non-hazardous compounds with the same accuracy as when using 6-pack assays. The distinct features of StopTox software include thorough data curation, rigorous external validation of models, transparent interpretation of models, ease-of-use, and intuitive interface. Models have been made available within our publicly accessible STopTox web portal (<https://stoptox.mml.unc.edu/>).

**Presentation:** Oral

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## Improving reproducibility and translation of animal research – an industry perspective

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Many preclinical data, including those from animal studies, cannot be reproduced due to methodological shortcomings, or issues with internal and external validity of research data, with sometimes far-reaching consequences on drug development and translation to humans. The low drug development success rate is an important concern in the scientific community in general and in pharmaceutical industry. To address these issues, we have formed an Innovative Medicines Initiative (IMI) consortium of scientists at leading universities, pharmaceutical companies, contract research organizations, technology companies and scientific associations, called the European Quality in Preclinical Data (EQIPD) consortium (<https://quality-preclinical-data.eu/>). Its goal is to investigate the variables that influence the quality of preclinical study data in drug research, to compare the quality of studies conducted by the pharmaceutical industry as well as academic research, to provide a training platform, and to develop a fit-for-purpose, preclinical quality management system. The EQIPD quality management system aims to be a tailored working solution that will improve the research process, increase data quality in a lean and efficient way and contribute to better trans-

latability. Here, the EQIPD quality management system and its utility will be described and compared with other quality management systems used in drug development.

**Presentation:** Oral

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## Regulatory feedback on the EU-ToxRisk (NAM)-supported Read-Across Advisory Document

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The EU-ToxRisk project is an integrated European “flagship” program driving mechanism-based toxicity testing and risk assessment for the 21<sup>st</sup> century. The project aims at promoting the use of “New Approach Methodologies” (NAMs) for regulatory chemical risk assessment. Alignment with the views of risk assessors from regulatory agencies therefore constitutes an important aspect of EU-ToxRisk. To this end, case studies are employed as a means of learning how NAMs can aid in addressing risk assessment questions of regulatory relevance. Specifically, the first round of EU-ToxRisk case studies explored ways in which NAMs can support read-across (RA<sub>x</sub>) for regulatory purposes. In this context, the EU-ToxRisk Regulatory Advisory Board (RAB) issued recommendations on how to report NAMs for regulatory assessment; some of the underlying thoughts were later incorporated in a reporting template for cell-based toxicological methods published by Krebs et al. (2019). From the experience gained in the case studies as well as from the feedback given by representatives from regulatory agencies such as ECHA, EFSA, RIVM, or BfR, an EU-ToxRisk RA<sub>x</sub> approach was formulated in a paper by Escher et al. (2019). As one of the “legacy products” of EU-ToxRisk, a “Read-Across Advisory Document” will be produced, to provide actors in industry and authorities with helpful practical advice on how to integrate NAMs into read-across strategies employed in the regulatory context. This presentation provides feedback on that document from a regulator’s perspective.

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**Presentation:** Oral

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## Classification of human reference data and their use for evaluating defined approaches for skin sensitization

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To support the evaluation of non-animal approaches for skin sensitization assessment, we collected data for 2277 human predictive patch tests (HPPTs; human maximization and human repeated insult patch tests) from more than 1700 publications. Each test was evaluated for reliability. Results from 2255 tests considered to be sufficiently reliable were further analyzed to better understand strengths and limitations of HPPT data and develop a strategy for using them to classify chemicals for their skin sensitization potential under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS). HPPTs are performed with single doses making identification of thresholds uncertain and difficult to use as reference data for test method validation. To overcome this challenge, classification criteria from the GHS were extended using a decision tree to partly resolve ambiguity in the results. If an individual chemical in the database had multiple discordant test results, a weight-of-evidence approach was used to obtain a single classification for the chemical. This classification approach was applied to a reference list of substances to support the evaluation of defined approaches (DAs) for skin sensitization proposed for inclusion in a new OECD guideline. Classifications were compared with those based on local lymph node

assay (LLNA) data, and the predictivity of the evaluated DAs vs. HPPT and LLNA was characterized. The results of this exercise are presented alongside some learnings about limitations of HPPT data and deficiencies in the current GHS approach. The entire HPPT database is publicly available via the NTP Integrated Chemical Environment (<https://ice.ntp.niehs.nih.gov/>) and may assist in future evaluations of alternative skin sensitization methods and development of new models. This project was funded with federal funds from the NIEHS, NIH under Contract No. HHSN273201500010C. The views expressed above do not necessarily represent the official positions of any federal agency.

**Presentation:** Oral

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## iPSC-derived human BrainSpheres: A multifaceted and powerful 3D model for neurotoxicity testing

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The current guidelines for safety evaluation of chemicals are based on highly time-consuming and expensive animal tests. However, animals differ from humans in their way to handle chemicals and there are ethical concerns regarding their use. Thus, the development and use of human-based models for toxicity testing is strongly encouraged. Due to the high complexity of the brain, complex cell culture systems containing the main brain cell types, allowing a maximum of cell-to-cell interactions and recapitulating the main neurodevelopmental processes, are required for the evaluation of the adverse effects of chemicals on the nervous system. Here we use one model that fits all these requirements: the human iPSC-derived cultures BrainSpheres (BS) comprised of electrophysiologically active neurons, astrocytes and oligodendrocytes able to form compact myelin sheaths around axons. To predict *in vitro* the unwanted effects of chemicals in humans, in particular after repeated exposure, distribution kinetics of these chemicals have to be established. The concentration-effect relationships depend on the activity of the chemical and the sensitivity of the target, but also on the distribution of the compound in the *in vitro* system. Under the MSCA training network “in3”, BS were exposed to diverse chemicals in an acute and repeated way, then *in vitro* distribution kinetics and neurotoxicity were assessed. Results after amiodarone exposure showed a nice parallel between its dose- and time-dependent



intracellular accumulation and neurotoxic effects. Astrocytes were the most sensitive cells, and a disruption of lipid metabolism was revealed by TempoSeq analyses across exposure scenarios. Exposure to paraquat affected mostly neurons, including the dopaminergic ones. TempoSeq analyses highlighted oxidative stress and estrogen receptor-mediated signaling pathways, the former being disrupted differently according to exposure duration. Altogether these data exemplify the versatility of the BS model, the importance of *in vitro* biokinetics and of omics data analyses for neurotoxicity testing.

**Presentation:** Oral

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### ***In vitro-in silico*-based assessment of species differences in kinetics: Towards harmonization of *in vitro* clearance studies**

*Jochem Louisse*<sup>1</sup>, *Nicole Pinckaers*<sup>1</sup>, *Sandra Coecke*<sup>2</sup>, *Ad Peijnenburg*<sup>1</sup> and *Ans Punt*<sup>1</sup>

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In the safety assessment of chemicals, safe levels in humans are generally based on animal toxicity data. Uncertainty factors for interspecies differences in toxicokinetics and toxicodynamics are applied to translate animal data to a safe dose in humans. Generally, default factors are used, i.e., a factor 4 and 2.5 to account for interspecies differences in toxicokinetics and -dynamics, respectively. If quantitative data on interspecies differences in toxicokinetics are available, one may deviate from the default uncertainty factor for kinetic differences, thereby refining the safety assessment. The present study aims to assess whether applying *in vitro* metabolism data and *in silico* physiologically based kinetic (PBK) modelling, can be used to estimate differences in dose-dependent internal exposure in rats and humans. *In vitro* clearance data obtained with rat and human primary hepatocytes were gathered from the literature (Louisse et al., 2020) and new data were obtained for a selection of chemicals in our own lab. These data were applied in our recently developed generic PBK modelling platform (Punt et al., 2021) to estimate relative differences in C<sub>max</sub> and AUC between rats and humans. A large variability in reported clearance values was identified, which partly relates to differences in applied study designs. Differences in input values largely affect PBK model predictions, indicating the need for harmonization of clearance methods. When applying median reported clearance values, predicted species differences in internal exposure for some chemicals differed more than the kinetic interspecies subfactor of 4, indicating that for these chemicals, deviation from the standard uncertainty factor can be considered. Overall, it is expected that gaining experience with PBK models for interspecies evaluations may facilitate the transi-

tion towards next generation (animal-free) risk assessment strategies where these models will become a critical tool to translate *in vitro* toxicity data into human dose- or potency information.

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**Presentation:** Oral

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### **Three R challenges in the breeding of GA rodents**

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Recently published EU statistics revealed that, in 2017, more than 5,5 million genetically altered (GA) animals are bred and not used further in approved procedures. Additionally, 642,832 animals were reported under the (regulated) category of maintenance of established GA lines of which about 20% develop a harmful phenotype. These figures show that a large number of GA animals can be found in research animal facilities and, moreover, a large proportion experiences pain, suffering or distress caused by the genetic alteration.

In Switzerland and EU member states, regulations and guidelines are in place to ensure welfare assessment of GA lines which require a classification of phenotype severity and an implementation of measures to reduce suffering and animal numbers. Experience with their practical application shows that uncertainties remain. For example, severity classification of harmful phenotypes still seems to be performed largely on subjective basis and, hence, severity classification is unharmonized. Furthermore, evidence-based research on severity assessment is scarce and it is not clear which observable or measurable parameters are of general use for meaningful welfare assessment.

The number of animals produced in rodent animal facilities is one aspect of reduction. A reduction is possible by making better use of statistics, but also by refining the methods for evaluating the results obtained with these animals. Strategy calculations are complex in breeding of GA rodents and can only be done with powerful software. Currently, no tool is available to calculate all breeding strategies and compare which will produce the minimum number of experimental animals. The project of the Institute for Laboratory Animal Sciences of the University of Zürich



in collaboration with MathYou in Berlin intends to offer the scientific community a software solution.

The presentation will emphasize current challenges of GA breeding with focus refinement of harmful phenotypes and reduction of animal numbers.

**Presentation:** Oral

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## The process for the deletion of ATT, TABST and LABST in India

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India has been at the forefront among Asian countries in considering the implementation of alternative methods to animal testing, and among the first to accept waivers to the Abnormal Toxicity Test. Regulatory authorities, human and veterinary vaccines manufacturers, and international non-profit organizations like HSI and HSI/India worked together to facilitate dialogue and the decision-making process to successfully delete ATT and are currently working to promote the deletion/implementation of the waivers for TABST and LABST. HSI/India is presenting the work done in collaboration with Indian stakeholders in the process for these important changes, highlighting the drivers and the remaining barriers to complete this process.

**Presentation:** Oral

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## An inventory of non-animal methods to study Alzheimer's and Parkinson's disease

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In light of the continued efforts to reduce, refine and replace animal testing in biomedical research (3R), we inventoried and evaluated non-animal methods currently in use for basic and applied

research on Alzheimer's and Parkinson's disease (AD/PD), the two most common and most studied neurodegenerative diseases.

Publications that appeared between 2013 and 2018, combining a specific mention of (a) AD or PD, (b) a relevant biological endpoint to said disease, and (c) a method or model system (e.g., "cell model", "comput\*" etc.) were retrieved. More than 13,000 abstracts were screened, of which approx. 70% were rejected, primarily because of the use of animal-based methods or models. The remaining fraction described studies that develop, optimize or apply non-animal methods.

For PD, methods based on human primary or stem cells represented the highest fraction (35%), followed by human/patient *ex vivo* tissue and body fluids (16%), biochemical assays (15%), human-derived immortalized cell models (14%) and computational/*in silico* methods (14%). For AD on the other hand, biochemical/cell-free models represented the most commonly used group of methods (27%), followed by human/patient *ex vivo* models (22%) and computational models (18%).

The resulting inventory of non-animal methods comprised 568 different models, with detailed information about context and use. Answers to central questions "Why?", "How?" and "What?", as well as qualitative information related to the status, relevance and potential of a given method were compiled. As such, the inventory allows for straightforward browsing of existing published methods, which aims to contribute to 3R knowledge sharing and their increased adoption and acceptance in neurodegeneration research and related fields.

In addition to the inventory itself, our literature screen provides a rich source of information to identify areas of focus and interest in relation to innovative methodological development for basic and translational research.

**Presentation:** Oral

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## Think big and scale down: Development of a "skin allograft on-a chip model" emulating immune cell-skin interactions

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Microphysiological systems enabling long-term co-culture of various human organ equivalents have become increasingly useful for preclinical systemic drug testing (Ewart et al., 2018). Currently, these systems lack integration of systemic immunocom-



petence and therefore do not generate human immune responses. This hampers their use for the evaluation of vaccines, adjuvants and immune cell therapies as well as the investigation of mechanisms of organ rejection. Therefore, we are aiming to reliably mimic immune cell behavior on our microphysiological HU-MIMIC platform.

Here, we present the development of a “skin allograft on-a-chip model” which combines human skin biopsies with allogeneic peripheral blood mononuclear cells (PBMC) to emulate mechanisms of skin rejection (Marino et al., 2016). To distinguish between responses initiated by the co-culture environment, respective monocultures were performed in parallel. Tissue and cell behavior were investigated by immunohistochemical staining, detection of cytokine release patterns and by characterization of the present immune cell populations at relevant time points. PBMCs could be tracked in the skin until the end of the nine-day co-culture. Interestingly, mainly T cells survived and were more antigen-experienced in the co-culture. In addition, only the co-culture showed increased levels of IFN-gamma, IL-2 and IL-17 from day seven onwards. Hence, our co-culture showed first signs of rejection and might also be suitable to investigate pathogenic mechanisms in chronic inflammatory diseases, e.g., psoriasis, or testing of novel therapeutic approaches in the future.

The next step will be the combination of our immune cell-skin co-culture assay with an *in vitro* generated 3D lymphatic matrix aiming to emulate the human lymph node functionality *in vitro* (Kraus et al., 2019). Therefore, we integrated a chip-compliant hydrogel including PBMCs on our platform. Additional results on these matrices will give insights into their cellular composition and micro-organoid formation as well as cytokine release patterns over a fourteen-day culture time.

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**Presentation:** Oral

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## Validation in a regulatory context – A EURL ECVAM perspective on principles, practice and progress

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Validation is intrinsic to the scientific process. Within the biomedical research domain, growing concerns about the reproducibility and applicability of scientific results, particularly those generated using animal models, has put the topic of validation at the center of recent scientific discourse. Within the regulatory domain, formal validation has typically been a requirement for new test methods proposed as internationally recognized guidelines for generating toxicological data used in the hazard and risk assessment of chemicals. In this context, the OECD defines validation to be “the process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose”. Assembling the evidence can be tackled in different ways, such as the “modular approach” proposed by the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM). Here, information on test definition, transferability, within and between laboratory reproducibility, predictive capacity, applicability domain and proposed performance standards can be gathered somewhat independently and can combine retrospective analysis of existing information with the prospective generation of new data, as appropriate. Moreover, it provides a flexible basis to design fit-for-purpose validation studies that address different objectives. With the emergence of Integrated Approaches to Testing and Assessment (IATA), based on using Adverse Outcome Pathways (AOP) to guide the optimal integration of *in vitro* and computational methods, validation needs to address both the methods and the IATA itself, including determining the applicability domain and identifying and characterizing sources of uncertainty. Here we review the requirements for validation and the underlying principles and describe how EURL ECVAM has evolved its validation practice to keep pace with scientific progress and emerging regulatory needs. In addition, on-going validation studies in the areas of skin sensitization, developmental neurotoxicity and endocrine disruption (thyroid) are described as practical illustration.

**Presentation:** Oral



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## In vitro approaches for skin sensitization of medical devices

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Skin sensitization, one of three the biocompatibility endpoints required for all medical devices (MD), is still based on *in vivo* approaches (ISO 10993-10). The need for nonanimal methods relies not only on ethical reasons but also on scientific reasons as the accuracy of the commonly used animal tests (GPMT, Buehler test, LLNA) barely reach 70% compared to human data. In 2012, OECD proposed an Adverse Outcome Pathway (AOP) for skin sensitization and several *in chemico* and *in vitro* assays have been validated to be used in testing strategies. However, these OECD assays concern neat chemicals and use mainly cell lines cultivated in 2D which are not easily adapted to medical devices extracts where potential leached sensitizers are diluted in polar and nonpolar solvents. The recent validation of *in vitro* skin irritation methods for medical device extracts demonstrated the added value of reconstructed human models (RhE). For skin sensitization, assays with RhE alone (Petry, 2018; Andres, 2017; Cotterez, 2018; Coleman, 2015) or in co-culture with 2D cells (Schellenberger et al., 2019) showed promising results for complex test systems.

Skin sensitization is under discussion by the experts of the ISOTC194/WG8 to evaluate how it would be possible to adapt and validate the testing strategies proposed for pure chemicals to the specific context of MD. Unlike the situation of skin irritation, the existence of quantitative data for skin sensitization generated from human (NOAEL) or animal data (EC3) will facilitate production of reference test samples to robustly and comprehensively evaluate adaptation of OECD methods to the context of medical devices products. This presentation will give an overview of different *in vitro* approaches for skin sensitization and will present the last results in medical devices context with methods based onto 3D models as test system.

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**Presentation:** Oral

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## Feedback from 8 years of training on alternative methods in industrial and academic contexts

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For more than twenty years EPISKIN has been an active player in the development of alternative methods, whether by providing experimental systems based on reconstructed human 3D tissues, by developing non animal methods (NAMs) or by supporting the implementation of these methods in industrial, academic and CRO laboratories. Validation of alternative methods and regulatory evolvments in many countries result in an increasing demand for support and training. In line with our commitment for dissemination of non-animal methods (NAMs), we created EPISKIN Academy in 2012 to (a) facilitate the deployment and acceptance of validated alternative methods and (b) prepare new generations of scientists and toxicologists to use these methods and to participate in the development of future ones

In order to respond to the diversity of needs we have developed a modular program ranging from a short awareness and demonstration of these methods to full theoretical and practical laboratory training leading to certification. Our presence on 3 continents has enabled us to build long-term partnerships with various public, academic and governmental actors in several countries. These collaborative approaches in education are best suited to reach the right audience and provide holistic solutions in which trainees can not only receive hands-on training in methods but also acquire the scientific and regulatory knowledge essential to the success of these approaches. In 8 years, EPISKIN Academy has trained several hundred students, scientists and toxicologists from public and private organizations. 280 have been certified on OECD methods of corrosion and skin and eye irritation (TG431, TG439, TG 492). EPISKIN Academy is committed to accompany today challenges and to



prepare the future of alternative methods in toxicology by engaging, whenever possible, longterm partnerships with institutional partners

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**Presentation:** Oral

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### Good Practice: Key experimental details to highlight when drafting your research article

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A paper draft is not only a preliminary version, but rather the conceptual scaffold on which the key experiments to demonstrate a hypothesis or validate previous conclusions are mounted. Aside from filling an accepted consensus list of sections, a good draft must rely on experiments that are both well executed and well designed. Each of the pieces that will build the final draft must provide a solid piece of evidence, easily reproducible. The aim of this workshop is to provide a theoretical background of what is required to take into account when drafting a research article, and will provide the participants with guidelines, and a short checklist to assess the solidity of their design.

**Presentation:** Oral

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### Perspectives on the use of high throughput profiling assays in next generation risk assessment

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Recently, numerous scientific and regulatory organizations across the world have proposed the use of data from New Approach Methodologies (NAMs) for supplementing and increasing the pace of chemical safety assessments as well as reducing the number of laboratory animals used in toxicity testing. NAMs are defined as any technology, methodology, approach or

combination thereof that can be used to provide information on chemical hazard and risk assessment that avoids the use of laboratory animals. As part of a shift towards NAMs, the next generation blueprint of computational toxicology at the U.S. Environmental Protection Agency advocates the use of non-targeted high-throughput profiling (HTP) assays for initial characterization of the biological activity of environmental chemicals. Ideally, such assays should: 1) be capable of being deployed in a high-throughput concentration-response screening format for identification of potency thresholds for perturbation of cellular biology, 2) provide high content data that can be used to identify putative mechanisms of action and 3) be capable of being deployed across a variety of human-derived *in vitro* cell models. To date, two high-throughput profiling assays have been identified by EPA that meet these criteria: high-throughput transcriptomics (HTTr) using targeted RNA-Seq and high-throughput phenotypic profiling (HTPP) using Cell Painting. This presentation will provide a broad overview of the EPA Comptox Blueprint and highlight progress on the use of HTP assays for screening of environmental chemicals, including experimental designs, laboratory and computational workflows, data management and analysis strategies relating to both of these assays. This presentation will also provide examples of how information from HTP assays can potentially be used in next generation risk assessment (NGRA) applications such as bioactivity to exposure ratio (BER) analysis for chemical prioritization, putative mechanism of action determination and read-across using chemical structure and biological profile similarity scoring methods. This abstract does not reflect USEPA policy.

**Presentation:** Oral

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### Culture of care and governance: Two sides of the same coin

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The European Directive (2010/63/EU) talks about a “climate of care” (recital 31). However, the term most widely used is “Culture of care”. “Culture” is an umbrella term encompassing the social behaviours and norms of a society as a whole and behaviours and habits of individuals of this society. Since 2016, there is a Culture of Care network. This network promotes a mindset and behaviour that continuously and proactively works to advance laboratory animal welfare and the 3Rs. To establish a culture of care within a research institute, all stakeholders need to be on board wholeheartedly and work towards a common goal. This relies on good governance, where strategic vision, accountability, and fairness are firmly rooted and based on sound ethical and moral standards. Both good governance and a culture of care are



required to provide the best for the animals and the science. The challenge remains to ensure that the “culture of care” extends to the animals at all times.

**Presentation:** Oral

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## The beyond animal testing index: How to assess your institute's contribution to the 3Rs?

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The transition to animal free innovation is an ambition with some urgency in the Netherlands and at the level of the European Union. Animal free innovation is not a new initiative. Those innovations are generated where science and technology meet. Sometimes as a result of purposeful researching and building, more often in a more serendipitous way. The beyond animal testing index (BATI) has been developed as a benchmark for research institutes involved in fundamental and applied research using animals to compare their respective contributions to developing and implementing animal free innovations and in a more general sense to the 3Rs based on the information already present in academic institutes in the Netherlands. The BATI is developed after the Access to Medicine Index, which benchmarks pharmaceutical companies according to their efforts making their medicines widely available also in third world countries. The BATI was designed and drafted in 2018. The draft BATI was presented to stakeholders, such as governmental and health funding organizations. Their feedback was included to produce the first version of the BATI. Currently a pilot among three academic medical centres is in preparation. Based on this pilot, further refinements of the BATI will be considered. As a benchmark tool, the BATI is expected to act as a stimulus for research institutes to learn and improve on their strategies towards the transition to animal free innovations, to identify gaps in the development of 3R technologies, and to monitor the effectivity of (inter)national policies.

**Presentation:** Oral

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## Towards a regulatory application of Caco-2 advanced intestinal barrier model

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It is a matter of fact that advanced *in vitro* models should be complex as required to reflect the *in vivo* situation but simple and defined enough to be reproducible and transferable. This implies that a careful definition of model parameters and/or experimental conditions is critical for their applicability to toxicological/pharmacological studies.

Tri-culture model of intestinal barrier of Caco-2 cells co-cultured with different intestinal cell types as muco-secretory cells (HT29-MTX) and lymphoblastoid cells (Raji B) was extensively utilized in evaluation of chemicals and nanomaterials (NMs) absorption. However, it still shows quite variability, which invalidate its wider use as *in vitro* model of intestinal barrier.

A pilot study to standardize and optimize Caco-2 tri-culture model is running at ISS, in the perspective of its application to NMs translocation studies. The study is finalized to develop an OECD Guidance Document on the definition of an *in vitro* approach for gastro-intestinal fate of ingested NMs.

Several model parameters were considered as insert pore dimension, microfold cell (M cell) induction and characterization, mucus production. Different endpoints of M cell phenotype induction on Caco-2/Raji B co-culture, such as transepithelial resistance (TEER) decrease, ZO-1 protein expression, and barrier permeability to FITC-dextran and fluorescent silica nanoparticles were evaluated. Mucus influence on silica nanoparticles absorption was also investigated.

Furthermore, to determine if a direct contact between the two cell types can better stimulate M cell induction, an inverted co-culture model was developed in which Caco-2 cells facing the basolateral compartment and Raji B cells were added to the apical compartment. Both models, normal and inverted, were able to induce M cells transformation in Caco-2, but the latter seems to be more performant.

The study results furnish a relevant contribute to move this three-culture model versus regulatory context.

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**Presentation:** Oral



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## Confronting publishing bias against *in vitro* approaches

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Publication of research is a necessary step in the dissemination and implementation of biomedical advances and plays a central role in the advancement of researchers' careers. Through the manuscript review process, editors and peer reviewers act as gatekeepers of biomedical research. This process is not unbiased, though. During the review of biomedical manuscripts, *in vivo* validation of *in vitro* experiments is often requested or required. Some journals explicitly state that manuscripts with *in vivo* animal evidence will be given higher priority for review over those with purely *in vitro* experiments. We argue this is a form of content-based bias. Whether implicit or explicit, bias against manuscripts reporting on *in vitro* methods is often unfounded and based on editors' and reviewers' preference, familiarity, or narrow expertise, leading to the evaluation of manuscripts not on merit (e.g., physiological relevance of the experimental model) but on irrelevant characteristics (e.g., a reviewer's preference for *in vivo* experiments). This is particularly troubling in the context of the transparency and translatability crises plaguing animal research. Bias against *in vitro* methods may contribute to additional animal experiments being performed for the sole purpose of appeasing biased editors and reviewers, by untrained laboratory personnel, and/or in a manner that lacks scientific rigor. Furthermore, this bias stands in the way of innovation and uptake of more physiologically relevant approaches such as human cell-based microphysiological systems and organoids, which have many advantages over the use of animals. Concrete steps to combat bias against *in vitro* methods may include broadening *in vitro* expertise on editorial boards and in reviewer pools, conducting anti-bias training, and establishing open peer review. Ultimately, a major shift in the perception of *in vitro* methods will be required for the advancement of ethical, effective biomedical research.

**Presentation:** Oral

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## Exploring 3D bioprinting technology for the development of complex reconstructed skin model with hair follicle structure and automation of the fabrication of hair follicle spheroids

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A variety of human skin models have been developed for applications in *in vitro* studies. Typically, these reconstructed skin models employ protein-based scaffolds along with human skin cells: fibroblasts and keratinocytes. A key limitation of these models is that they still fail in recapitulating the cellular and microenvironmental complexity, such as the presence of vasculature, multiple cell types (e.g., melanocytes, neural and immune skin cell) and adnexal structures (e.g., hair follicles, sebaceous and sweat glands), that are representative of human physiology.

In parallel, 3D bioprinting technology has been gaining attention as a platform for tissue engineering given the possibility for precise cell positioning, flexibility, reproducibility, and high-throughput production. We have explored these advantages for the development of two models of the human hair follicle. For the reconstructed skin model, a bioink containing dermal papilla cell (DPCs) and human umbilical vein cells (HUVECs) was precisely printed within the gelled dermis. These cells formed spheroids inside the dermis which were enveloped by keratinocytes and melanocytes migrating from the epidermis through the vertical opening left by the nozzle. The resulting model contained a hair follicle-like structures whose morphology and biomarker pattern mimics that of the native tissue. Additionally, we have developed a hair follicle spheroid model formed by a core of DPCs and HUVECs (step 1) enveloped by a sheath of epithelial cells (step 2). The resulting spheroid, generated in an automated, precise and reproducible manner by 3D bioprinting, also resembled the structure of the native hair follicle and could potentially be used for high-throughput screening of substances.

The development of reconstructed skin models with increased complexity that better mimic the native tissue can have an important impact on the diversification of *in vitro* models available for safety and efficacy assessment of chemical compounds.

**Presentation:** Oral



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## Applications of brain-model technology to study chemical induced neuro(developmental) disorders

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There is concern that environmental exposures contribute to the increased prevalence of neurodevelopmental and neurodegenerative disorders such as autism, ADHD, Parkinson's and multiple sclerosis. However, very few compounds have been identified as (developmental) neurotoxicants due to limitation in current *in vivo* testing guidelines. Moreover, there is a lack of effective drugs to treat neurological disorder despite decades of research using current animal models in drug discovery. Pharmaceutical companies are desperately seeking human-relevant alternatives as more than 90% of drugs fail in clinical trial.

We have developed a reproducible iPSC derived human 3D neural cell model, comprised of differentiated neurons (glutamatergic, dopaminergic and GABAergic neurons) and glial cells (astrocytes and oligodendrocytes) (Pamies et al., 2016). The model has shown to be relevant for key cellular processes involved in neurodevelopment including proliferation, differentiation, apoptosis, synaptogenesis, intracellular signaling, and network function. In addition, it presents unique features as it has shown *de novo* myelination. The failure to form or maintain myelin can disrupt neuronal signal transmission or trigger degradation of axons and can induce severe neurological symptoms and disorders such as multiple sclerosis, amyotrophic lateral sclerosis and periventricular leukomalacia. The human model has been exposed to pesticides, environmental contaminants, pathogens, drugs and flame retardants to induce (developmental) neurotoxicity and disease assessed by omics and high content imaging approaches (Pamies et al., 2018; Abreu et al., 2017; Zhong et al., 2020). The model has the potential to become a human-relevant alternative to animals in toxicology and disease.

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**Presentation:** Oral

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## Assessment of acute stress and anxiety by infrared thermography

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Evaluating emotional states in animals often relies on behavioral indicators, thus being vulnerable to subjective biases and other factors, including observers' experience. Therefore, more objective methods for assessing mental states in animals are needed. Body temperature variations have been shown to be valuable physiological indicators of acute stress or anxiety, namely by the "hyperthermic stress response" associated with the fight or flight response, and observed in multiple homeothermic species, from mice to humans. Stress-induced hyperthermia can hence be used as an objective measure of the anxiogenic effect of stressors in laboratory animals – from environmental factors to common procedures – to inform the choice of the least stressful methods, and to assess the efficacy of refinement measures.

Since the restraining necessary for measuring temperature by traditional methods is itself stressful, our lab has studied how infrared thermography can be used for non-invasive thermal assessment of laboratory animals. We have established mean body surface temperature (MBST) as a robust parameter for monitoring temperature variation from thermal images of group-housed, freely moving mice, as compared to the eye or tail (Gjendal et al., 2018). Following this, we developed "ThermoLabAnimal", a software application that automatically removes the thermal background from images and detects and provides MBST of individual animals, even when group housed, if not overlapping (Franco et al., 2019) Our approach, which may be applied to other species and scientific purposes (also being studied in our lab, such as giving insight to pathophysiology of disease, and possibly signaling humane endpoints) has the advantages of allowing thermal assessment of laboratory animals with minimum impact on physiology or behavior, while eliminating observer errors and biases.



This talk will present both past and novel results from our research group on the use and validation of infrared thermography for assessing stress and anxiety in laboratory mice.

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**Presentation:** Oral

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## Animal-free testing of cell-based medicinal products

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Cell-based medicinal products (CBMPs) consist of viable cells of human origin. Different animal species display different cell surface markers, activation factors, expression pattern of specific genes, and possess distinct immune system components making testing for toxicity challenging.

A project was launched under auspices of the Ministry of Agriculture, Nature, and Food Quality of the Netherlands, with the objective to study the possibility whether CBMPs could be developed safely for human use without animal testing.

By analyzing CHMP Scientific Advice reports written for 84 CBMPs in 2013 to 2019, our research revealed that approximately 70% of human cell products were tested in animals for toxicity. Only 5~12% of total products were tested with animal-free methods. No animal toxicity studies were performed with dendritic cell-based products. The performed toxicity studies were general toxicity (55%), immunogenicity (29%), safety pharmacology (19%), and a very low number of reproductive toxicity studies (2%). Animal models were roughly divided into rodents (mice or rats), large animals (pigs, sheep, goats, horses, and dogs), and non-human primates.

When analyzing the safety package of products without *in vivo* toxicity studies, we found that the informativeness of the safety package predominantly relied on already available clinical experience and subsequently on *in vitro* safety studies.

The observed adverse effects in the *in vivo* safety studies that were most commonly discussed were pulmonary thrombosis, effects caused by cell entrapment due to high doses, immunological effects, and effects that were difficult to interpret.

Thus, clinical data played the most crucial role in terms of product safety assessment. We envisage that a gradual shift toward animal-free safety testing of CBMPs will be the future trend in the development of such products.

**Presentation:** Oral

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## Regulatory consequences of the validation of replacement *in vitro* toxicity and antigenicity assays for *Clostridium septicum* vaccine antigens

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In 2016, 14 manufacturers and public sector control laboratories were enrolled into an international study run under the common aegis of the EDQM and the EPAA. The study allowed the validation of Vero cell-based assays as alternatives to the mouse tests currently in use for in-process quality control of *Clostridium septicum* vaccines (toxicity: Minimum Lethal Dose, antigenicity: Total Combining Power). The results demonstrated that optimized Vero cell-based assays represent credible toxicity and antigenicity indicators as alternatives to the corresponding *in vivo* methods. Implementation of such cell-based testing for other cytotoxic antigens, using this study as a model, could ultimately result in large reductions of animal usage in the quality control of veterinary vaccines (Daas et al., 2020; Behr-Gross et al., 2021). In consequence, the experts of the group 15V of the European Pharmacopoeia launched the revision of monographs *Clostridium septicum* vaccine for veterinary use (0364), *Clostridium novyi* (type B) vaccine for veterinary use (0362) and *Clostridium perfringens* vaccine for veterinary use (0363). The revised texts were submitted to public enquiry from July to September 2020. The outcome of the public enquiry will be presented and discussed – together with the practical information obtained during the collaborative study and from a large field enquiry – at a workshop in Strasbourg (9-10 March 2021). In an effort to foster international harmonization for the implementation of the replacement methods, the workshop jointly supported by the EDQM, EPAA and JRC/EURL ECVAM will be open to manufacturers and regulators from all over the world.

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**Presentation:** Oral

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## **In-silico trials for drug safety and efficacy assessment**

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Preclinical assessment of drug-induced cardiotoxicity is still a challenge for pharmaceutical industry, which is in need of new tools for its accurate and early identification (Lavery et al., 2011). *In-silico* trials using multiscale biophysically detailed human-based models have proven to be reliable as a new tool for pro-arrhythmic risk assessment, through the integration and augmentation of large and heterogenous clinical and experimental data with the most advanced computational and engineering techniques (Lancaster and Sobie, 2016).

Here, we present the most recent breakthroughs of our research (Passini et al. 2017, 2019), with a particular focus towards the replacement, reduction and refinement of animals in research and clinical translation.

Computer simulations were conducted using a new computational model for human ventricular electrophysiology (Tomek et al., 2019), designed, calibrated and validated against a wide set of experimental datasets and protocols. A population of models was designed to reproduce biological variability and used to test 40 reference compounds with a wide range of actions on cardiac ion channels. Drug-induced changes in electrophysiology and contractility of human ventricular cardiomyocytes were investigated at several concentrations and used to predict drug safety and efficacy. Several cellular properties were evaluated as biomarkers.

*In silico* drug trials successfully predicted drugs with clinical risk of drug-induced Torsade de Pointes (a ventricular arrhythmia), achieving 90% accuracy. The electromechanical window, accounting both for electrophysiological and contractility changes, was identified as the most reliable biomarker, even at low drug doses. Drug-induced electrophysiological effects were also in line with the experimental evidence for all drug classes.

*In-silico* drug trials showed higher accuracy than animal *in vitro* and *in vivo* models for drug-induced pro-arrhythmic cardiotoxicity. They are a powerful tool, already implemented in ma-

ny pharma companies to reduce time, costs and the use of animal models during the preclinical drug development.

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**Presentation:** Oral

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## **Advancing IATA in European chemicals regulatory decision-making**

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The European Union (EU) has over 30 pieces of key legislation to regulate a vast range of chemicals used in a multitude of sectors. Apart from facilitating the EU's single market and supporting industrial innovation and competitiveness, the EU's chemical acquis has also the important goal of protecting human health and the environment. A key provision in EU legislation is its continual adaptation to remain efficient and relevant. This includes the up-take of scientific and technical advances in hazard and risk assessment practice, with a strong preference for new approaches based on non-animal methods. Apart from societal pressure to move away from animal testing, there is also a growing scientific impetus for more human-relevant approaches such as complex *in vitro* methods using human-derived cells and tissues and computational modelling of human metabolism and biokinetics. However, as the tools become more sophisticated, solution-providers are challenged with how to optimally combine novel experimen-



tal and computational methods in a way that delivers the information needed for regulatory decision-making. To this end, the EU contributes heavily to OECD efforts to establish the Integrated Approaches to Testing and Assessment (IATA) framework and its primary aim of bringing non-animal methods to bear in regulatory domains. As scientists, end-users and regulators engage in developing and applying IATA in different risk assessment contexts, a number of issues are being grappled with. For example, how to strike the right balance between flexibility on one hand, to embrace the rapid evolution of the scientific toolbox, and formality on the other hand, motivated by expectations for validation and standardization to support implementation. Attention also needs to focus on understanding how best to characterize sources of uncertainty within IATA and how to go about establishing their scientific credibility to ensure acceptance and uptake.

**Presentation:** Oral

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## **In vitro methods to assess thrombogenicity of medical devices and materials: Effects of donor specificity**

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Thrombosis and inflammation are responses of blood components to any foreign body. With a few exceptions these responses should be kept to a minimum.

Implant materials are being tested extensively before clinical use with random fresh animal or human blood, with the assumption that animal species and different human donors show a comparable response to a certain type of material, which resembles the response of an implant recipient (Blok et al., 2016, 2019).

However, thrombotic and inflammatory reactions are highly different between individuals, resulting in either tolerance or in unacceptable high levels of activation with the same material. In particular, the extent of thrombosis shows marked differences between individual, which is shown by macroscopic evaluation (Reviakine, 2017).

This work aims to demonstrate the effect of individual responses on activation biomaterials, to quantify deposition of thrombotic elements by sensitive surface markers and to see if circulating blood activation markers correlate with blood element deposition. By using an *in vitro* closed loop circulation model human blood could be tested multiple times on multiple materials in one experiment (Engels et al., 2016).

We found that response of blood from a certain donor was highly reproducible but results of platelet and fibrin deposition of different donors was up to three-fold different on the same experimental day.

Many implant procedures are elective, which allows a screening protocol to be performed in advance. This protocol should be performed with a small amount of blood from the patient in an *in vitro* test system with a test object having a surface similar to the implant material. By intensive contact of blood with material data can be obtained in a short period of time. The outcome of the tests could result in use of another type of material.

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**Presentation:** Oral

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## **Human skin stem cell-derived hepatic cells as a tool for toxicity testing and drug development**

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Human skin precursors (hSKP) are adult stem cells that can be cultured *in vitro*. hSKP can differentiate towards hepatic cells (hSKP-HPC), expressing a mixed phenotype of mature (ALB) and immature (EPCAM, NCAM2, PROM1) hepatocytes.

Their applicability for hepatotoxicity testing was evaluated by exposing the cells to reference compounds. Acetaminophen-exposed hSKP-HPC predicted drug-induced “liver damage/necrosis”, comparable with observations made for patients suffering from liver toxicity caused by acetaminophen overdose (Rodrigues et al., 2014). Exposure to sodium valproate resulted in a drastic increase of intracellular triglycerides and a compromised lipid metabolism similar to cellular responses in the liver of patients suffering from hepatic steatosis (Rodrigues et al., 2016). Furthermore, amiodarone provoked the formation of lamellar bodies and accumulation of phospholipids, typical features of



phospholipidosis. This was accompanied by dysregulation of lipid metabolism- (SCD1, FASN, APOB) and lysosomal activity- (AP1S1, PLA2G15) related genes, accentuating the modulation of key mechanisms involved (Natale et al., 2018).

Recently, hSKP-HPC were also used to model non-alcoholic steatohepatitis (NASH), a severe liver condition characterized by steatosis and liver inflammation and for which no drug is currently available (Boeckmans et al., 2018). Exposure of hSKP-HPC to factors associated with the development of NASH (fatty acids, insulin, glucose and inflammatory cytokines) induced intracellular lipid accumulation and secretion of inflammatory chemokines (CCL2, CCL7, CCL8, CXCL5). Furthermore, “hSKP-HPC NASH” showed activation of gene classes related to “chemotaxis” and a similar transcriptional signature as observed in human NASH liver samples. Using this model, the anti-NASH properties of elafibranor, a clinical phase III compound, were investigated. Elafibranor restricted the increased lipid load and diminished the secretion of inflammatory chemokines. Gene expression data also suggested that elafibranor inhibits chemotaxis in a NF- $\kappa$ B-dependent manner (Boeckmans et al., 2019).

Overall, hSKP-HPC represent a valuable, pragmatic human-based *in vitro* system for preclinical hepatotoxicity assessment and anti-NASH drug development.

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**Presentation:** Oral

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## Applicable legislation for non-animal approaches in the food system

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Methods that can be used in toxicology and safety assessment are changing towards an increased application of non-animal approaches. Members of the International Life Sciences Institute (ILSI) Europe have formed an expert group to review possibilities, opportunities and challenges for the use of non-animal testing strategies in food safety research, which can ultimately be used in support of regulatory submissions for pre-market authorization.

The presentation will discuss the options for operators in the food industry on the basis of the EU-legal framework. The horizontal Directive 2010/63/EU on the protection of animals used for scientific purposes on the one hand makes compliance with the 3Rs principle a legal obligation in the EU since it came into force in 2013. Non-animal alternative approaches to an existing animal method must be used if “another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognized under the legislation of the Union”. On the other hand, compliance with EU-food legislation in order to ensure that food products and their ingredients are safe for consumers as well as for the environment at least currently still requires manufacturers to conduct some animal tests. Information from non-animal strategies can increasingly be used as part of a Weight of Evidence assessment and for risk prioritization. The presentation will invite discussion how the acceptability of non-animal methods or approaches in the food sector could be accelerated.

**Presentation:** Oral

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## Use of NAM under REACH and globally

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EU REACH (Registration, Evaluation, Authorisation and restriction of Chemicals), is based on the utilization of OECD (Organisation for Economic Co-operation and Development) Guideline Studies for classification and risk assessment, which, together with exposure considerations leads to risk management in the supply chain and/or regulatory risk management.

The test guidelines are the basis for the “Globally Harmonized System of Classification and Labelling of Chemicals (GHS)”. This system addresses classification of chemicals by hazard types and proposes harmonized hazard communication elements. It aims at making this information available to enhance the protection of human health and the environment.

In REACH, the use of existing information, waiving, as well as the possible utilization of “alternatives”, puts animal testing as a last resort. Many of the attempts by industry to fulfil the information requirements without testing, in particular for systemic toxicity, fail to meet the legal and scientific requirements, which will be elaborated.

As New Approach Methods (NAM) become available, they are integrated in the regulations, predominantly through the OECD programme. The NAMs that are integrated into the test guidelines programme, provide either specific (additional) information of toxicological interest and/or replace existing (animal) tests. For more complex systemic toxicological endpoints, replacement with NAMs seems challenging.



ECHA (European Chemicals Agency) is an active participant to Accelerating the Pace of Chemical Risk Assessment (APCRA). APCRA is a government-to-government initiative to promote collaboration and dialogue on the scientific and regulatory needs for the application and acceptance of NAMs in regulatory decision making. APCRA began in 2016 with a workshop designed to bring together regulatory agencies from around the world to discuss the practical application of NAMs to chemicals management. Until now, APCRA organized 4 workshops and initiated international collaborations on various case studies. This session will give an overview of the progress made so far.

**Presentation:** Oral

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## Remove ATT, TABST & LABST. The journey of how they became obsolete

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The general safety tests like the abnormal toxicity test (ATT) in mice or guinea pigs for human vaccines and the laboratory animal batch safety test (LABST) and target animal batch safety test (TABST) for veterinary vaccines are a key priority for global harmonization of legal requirements. Vaccines are produced for the global market, and discordance of legal requirements for their approval and market authorization as well as batch release testing greatly hamper the minimization of regulatory testing and related animal use. However, important progress has been made over the years towards agreement at global level that certain animal tests, namely the general safety tests have become obsolete thanks to the introduction of modern vaccine development with validation of the manufacturing process, advanced in-process controls, and product release testing complying with international standards. This presentation will highlight important aspects of this journey, which besides scientific reasoning only required a thorough exploration of the reasons for prevailing interregional differences in quality testing requirements and the building of trust and confidence.

**Presentation:** Oral

716

## Replacing the need for bovine blood products in early stage optimization of cardiac assist devices: Improving the international standard

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Artificial organs for circulatory support are lifesaving systems that support or replace the function of failing organs in critically ill patients. While these devices enable complex lifesaving interventions, increasing evidence implicates sub-optimal system design is likely to cause blood damage (not identified in earlier pre-clinical tests). Development processes of artificial organs are limited by international standards that recommend the use of bovine blood for predicting success of human life-support systems. Thus, we aimed to compare the suitability of human and bovine blood for artificial organ haemocompatibility testing.

Human blood was sourced, tested, and compared to data obtained from previous bovine blood studies for haematological and rheological parameters, including specific assessment for mechanical sensitivity of blood cells and high-shear tolerance.

Haematological assessment identified that when compared with bovine blood, human blood contains: ~25% more blood cells  $\cdot \mu\text{L}^{-1}$ ; erythrocytes with ~25% larger diameter, ~50% larger volume and surface area, and 10-15% less cytosolic haemoglobin (implicating substantially decreased cytosolic viscosity). Rheological profiles were also identified to be drastically different; human plasma is approximately half the viscosity of bovine plasma; however, as bovine erythrocytes do not aggregate, low-shear whole blood viscosity is markedly increased in human blood. Further, while human erythrocytes are substantially more deformable than bovine, human erythrocytes exhibit far greater susceptibility to shear-induced damage (i.e., half the strength of bovine blood).

Due to inherent biological differences that exist between human and bovine blood, it is likely that current bovine recommendations have resulted in the development of nonrepresentative models of blood-device compatibility. To improve the outcomes and quality of life of patients receiving artificial organ therapies, future devices must be designed, tested, and optimised for humans; bovine blood is a poor model of human tissue and should not be used as a surrogate.

**Presentation:** Oral

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## ***In vitro* coculture model (h-CLAT/RHE) composed of THP-1 cells and 3D reconstructed human epidermis to assess activation and maturation of dendritic cells**

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The development of non-animal-based assays (NAMs) for testing sensitization potential and potency of challenging materials with pronounced lipophilicity (e.g., fragrances or pharmaceutical actives) or polymers (e.g., medical devices or transdermal therapeutic systems) is an increasingly important aspect. Innate immune cells but also structural cells, for instance dendritic cells (DC) and keratinocytes are receptive to signals from their environment and interact with each other. Therefore, we developed a co-culture model (h-CLAT/RHE) consisting of the combination of the OECD validated Human Cell Line Activation Test (h-CLAT) and a reconstructed human epidermis model (RHE) (Schellenberger et al., 2019). We placed THP-1 cells underneath the RHE, to be close to the *in vivo* situation, e.g., stratified layers of keratinocytes providing physical and chemical barrier properties. Furthermore, the integration of RHE allows the application of test materials to the skin surface, which enables testing of a wide variety of challenging materials and material states.

As endpoints, we analyzed the cell surface expression of the costimulatory molecule CD86. The adhesion molecule CD54 on collected THP-1 cells by flow cytometry. We proved the feasibility to use the selection criteria for hazard prediction of h-CLAT, namely a relative fluorescence induction of 1.5 and 2.0 for CD86 and CD54, respectively. In total, we identified 12 chemicals as skin sensitizer (including weak, moderate and strong sensitizer). Two molecules could be tested as sensitizers, which were negative in submerge coculture assay (COCAT) of THP-1 and HaCaT keratinocytes (Hennen and Blömeke, 2017; Eskes et al., 2019).

In conclusion, we showed with a limited set of skin sensitizers that the implementation of keratinocytes using RHE with selection criteria of the h-CLAT is possible. In addition, the set-up may be able to overcome issues of lipophilicity, solubility and testability of challenging materials in skin sensitization.

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**Presentation:** Oral

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## ***In silico* modelling as a way to prioritize experiments and reduce experimental testing for osteoarthritis drug target discovery**

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The European Commission published a strategic document to shape the future research and innovation investment, which repeatedly mentions digital tools as an asset for healthcare (<https://www.vph-institute.org/news/2019-activities-and-achievements.html>; <https://ec.europa.eu/research/pdf/horizon-europe/annex-1.pdf>), in line with the potential of *in-silico* research to achieve the 3Rs. Indeed, computer modelling may help study various complex diseases and therapeutic strategies while decreasing the experimental burden. Here, we present several aspects of our *in-silico* research in which we implemented computational solutions to understand the complexity of the osteoarthritis (OA) disease. These same tools are used to identify potential therapeutic strategies, through endogenous (pharmacological) or exogenous repair (tissue engineering).

First, we aimed at extracting as much information as possible from published transcriptomics datasets by combining them. We gathered data from several mouse OA models and integrated them while removing the variation solely due to the technical batch effects. With unsupervised clustering, we showed that the biological information remained intact. The gain of information provided by the microarrays merger enabled us to infer a gene regulatory network (GRN) using machine learning algorithms.

The GRN complemented a biochemical interaction map of intracellular processes involved in cartilage cell (chondrocyte) differentiation. It resulted from the accumulation of decades of mechanistic knowledge. Not only did this map represent a new knowledge base, it was also translated it into a mathematical model for subsequent analyses. It enabled *in-silico* testing of hypotheses about intracellular control of chondrocyte fate decision.

Combining both approaches lead to a comprehensive modeling tool to screen potential endogenous and exogenous therapies for osteochondral diseases. We established an *in vitro* semi-high throughput assay to validate the *in-silico* model predictions



about promising drug targets for OA. We highlighted a synergistic effect between two targets via that *in-silico in vitro* approach. *A priori* testing of conditions with this modelling approach could refine the drug discovery pipeline.

**Presentation:** Oral

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## Political campaigning: Where scientific and ethical arguments meet public policy

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What drives change? Does the elimination of animal research and testing rely more on scientific progress or changing public attitudes? At EU level, politics is at least as important as science – not least because legal requirements are helping shape science funding and policy decisions. Within biomedical research new technologies allow previously unimagined opportunities to study human disease in humans, and in regulatory testing the relevance of animal methods is increasingly questioned. But other scientific and policy developments are also important. Studies establishing the sentience of certain invertebrates led to new legal protections. Project evaluations as defined by Directive 2010/63/EU must consider ethical aspects of animal use, and knowledge of animal sentience and cognition is increasing – at some point, might an EU project evaluator consider that just as rodents should be housed in groups because they are social animals, maybe their use should be entirely avoided for more or less the same, or similar, reasons? Sadly, though, these developments are not enough. While many scientists and regulators remain open to change, conservatism and inertia present obstacles even when replacement of animal use is not only technically possible but scientifically desirable. Within this context, it is essential for animal advocates to check that the right strategies are employed. Should we favour advancing science or political gain? Which stakeholders should we work with, and is there anything we could or should be doing that is not currently prioritised? How far are we able to manage threats, such as those posed by other stakeholders successfully campaigning for increases in animal test requirements – and, as ever, how do we expose the scientific limitations of animal use when many of those involved have an interest in defending the *status quo*?

**Presentation:** Oral

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## IATA as the nexus of traditional bioassay and new approach methods data in human health assessment

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Identification of human health effects associated with potential exposures to 1000's of data-poor commercial and environmental chemicals is significantly limited by the lack of traditional *in vivo* hazard and dose-response information however, absence of information is not equivalent to absence of risk. As such, efficient and timely chemical assessment in a data-poor environment may be realized through a paradigm shift in which extant toxicological information, albeit limited at times, is integrated with New Approach Methodologies (NAM)-based data in an Integrated Approach to Testing and Assessment (IATA). IATA is a problem formulation based pragmatic approach to assembling and evaluating information from diverse chemical and biological data streams, taking into account the acceptable level of uncertainty associated with a given decision context. IATA may include data-mining and systematic review of existent *in vivo* toxicity information, -omics data, *in vitro* or *ex vivo* bioactivity, and/or structure-activity such as QSAR and read-across, to answer questions in a fit-for-purpose assessment application or regulatory decision context. IATA applications may range from identification of sufficient traditional bioassay information to make a decision, with NAM used in a data-gap filling mode, to a complete lack of traditional bioassay information with NAM data working in concert to serve as the basis for hazard identification and dose-response assessment. This session will provide insights into IATA application and contextualize the scoping of information for various environmental chemical evaluation foci.

The views expressed in this abstract are those of the author and does not necessarily represent the views or policies of the United States Environmental Protection Agency

**Presentation:** Oral

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## Opportunities for use of IATA in Canada's chemicals management plan

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Chemicals management faces global challenges including aggressive assessment mandates, a rapidly changing chemical

landscape to new and more complex chemistries, a large proportion of chemicals with little toxicological data available for prioritization and assessment and increasing pressure to reduce and eliminate animal testing. Under Canada's Chemicals Management Plan (CMP) there has been a progressive increase in the exploration and integration of computational tools and New Approach Methods (NAM), including Integrated Approaches to Testing and Assessment (IATA), to support specific regulatory applications and defined decision contexts. A barrier to the adoption of some NAMs in certain regulatory contexts is the lack of harmonized, internationally recognized guiding principles. To address this need, collaborative case studies conducted under programs such as the Organisation for Economic Co-operation and Development (OECD) IATA project (Webster et al., 2019) and Accelerating the Pace of Chemical Risk Assessment (APCRA) (Kavlock et al., 2018) are exploring the application and interpretation of these new approaches and emerging data. As confidence continues to grow, the IATA framework is being examined to support priority setting, problem formulation and risk assessment activities. Examples of recent advancements in the application of IATA under the CMP will be presented. Key features include the use of computational approaches for analogue selection for read-across, data collected from traditional and NAM sources, and the development of workflows to generate predictions specific to the chemical space or groupings of interest. Based on experience to date, the use of IATA promises to provide a framework for the development and application of fit for purpose approaches that incorporate evolving science to accelerate screening, priority setting and chemical risk assessment in Canada.

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**Presentation:** Oral

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## Scientific validity of non-animal-derived antibodies

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Affinity reagents are binding molecules that have a high specificity for their unique target (antigen). They are crucial tools for research, diagnostics, therapeutic and regulatory applications. Based on their recognition properties and binding specificity, protein-based antibodies are currently still the most important tools for the specific detection of proteins or other molecules. Animals are still widely used for the development and production of monoclonal and polyclonal antibodies as well as other affinity reagents despite the availability of alternative non-animal technologies for more than 20 years. In line with the legal requirements of EU Directive 2010/63/EU on the protection of animals used for scientific purposes, animals should not be used in procedures where a non-animal alternative that provides the same or higher level of information exists. For this reason, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) mandated its Scientific Advisory Committee (ESAC) to review the available evidence and deliver an opinion on the scientific validity of antibodies and non-antibody affinity reagents produced using animal-free technologies. The review focused on non-animal-derived antibodies generated by phage-display technology. Taking into consideration the available evidence, the ESAC concluded that non-animal-derived antibodies are mature reagents generated by a proven technology, being able to replace animal-derived antibodies in the vast majority of applications. They have no general or systematic disadvantages with respect to affinity, stability, shelf life and specificity, and offer significant scientific and economic benefits. Based on the ESAC Opinion, EURL ECVAM issued its own recommendation that the provisions of Directive 2010/63/EU should be respected and the use of animals for the development and production of antibodies should no longer be authorized in the absence of robust and legitimate scientific justification (Barroso et al., 2020). An overview of the ESAC findings and of the EURL ECVAM Recommendation on non-animal-derived antibodies will be presented.

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**Presentation:** Oral



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## A 3D-printed microplate insert for high-throughput and ultra-long term high resolution imaging of live human brain organoids: A new platform to replace animal models in brain cancer research

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In contrast to animal models, human brain organoids replicate the architecture and neuronal composition of the human brain, which is useful for the study of human brain development and disease. However, current methods for human brain organoid culture are low-throughput and not suitable for long-term high-resolution imaging, which is required to study developmental processes and disease progression within physiologically relevant time frames (i.e., days, weeks, months). Here we demonstrate the utility of 3D-printed microplate inserts, which enable the scaling up of brain organoid culture and the growth of brain organoids in pre-defined XYZ coordinates. Together, these innovations facilitate high-resolution and high-throughput imaging of brain organoids over long periods of time (up to 2 months). We show that brain organoids grown using 3D-printed microplate inserts do not significantly differ in terms of gene expression, tissue architecture, and growth rates from brain organoids obtained using standard protocols. Finally, we applied this technology to visualize the growth of patient-derived glioblastoma stem cells as tumours within healthy brain organoids. Overall, this new bio-engineering platform constitutes a significant advance that permits high-throughput studies of several brain diseases using organoids and high-content phenotypic imaging, thereby replacing the use of animal models in brain cancer research.

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*Australia; the Neurosurgical Research Foundation; the Cancer Council SA Beat Cancer Project; the Beat Cancer Project and Health Services Charitable Gifts Board, the Australian Research Council (ARC), a Premier's Research and Industry Fund grant provided by the South Australian Government Department for Innovation and Skills and the Medical Advances Without Animals trust (MAWA).*

**Presentation:** Oral

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## Automating multi-organ-chip assays and analysis for improved standardization and reproducibility

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Microphysiological systems (MPS) are designed to mimic human organ function and physiology *in vitro* at the smallest biologically acceptable scale. These systems pose an alternative approach to laboratory animals in academic and pharmaceutical research where they are envisioned to improve the current drug development process. However, the performance of long-term *in vitro* assays using MPS requires high manual effort and work force. This hampers the adaptation to industrial needs, where reproducibility and standardization are essential demands. In addition, automation would facilitate the use of MPS for regulatory purposes (Marx et al., 2020).

Here we present our fully automated HUMIMIC AutoLab system for the standardized long-term cultivation of TissUse's Multi-Organ-Chips. The system can operate 24 Multi-Organ-Chips simultaneously, providing systemic pulsatile media circulation and execution of all liquid handling steps under sterile conditions. For proof of concept, we transferred our well-established manual co-culture assays with organ models such as liver, skin, intestine and bone marrow organoids on to the system. To this end, the HUMIMIC LabOS software enables the intuitive input of all assay information and provides many data analysis features such as pattern recognition for spheroid tracking or machine learning algorithms supporting, e.g., virtual staining. All handling procedures including media change, substance application and sample extraction are executed automatically. In addition, the system allows for routine microscopic analyses such as bright field imaging and fluorescence measurements. A high comparability to the manually conducted assays, which were performed in parallel, was achieved. Data will be presented on assay specifications including compound treatment regimens, metabolic analysis, gene expression data and immunohistochem-



ical staining confirming organoid viability and functionality.

While requiring less personnel resources and enabling a more standardized and reproducible operation of the Multi-Organ-Chips, our AutoLab will also make it easier to run even more complex culture strategies leading to a near physiological organ maintenance.

#### Reference

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**Presentation:** Oral

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## Advanced human cell models to support safety assessment of bi-specific antibodies

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The use of advanced human cell models presents a great opportunity to significantly improve drug development processes and to increase probability of technical success for the right candidates to enter clinical testing. This approach allows 1) the study of human disease biology for target and biomarker identification, 2) selection of the most promising drug molecules based on pharmacology, metabolism and safety, 3) testing drug combination regimens and 4) addressing patient population specific effects by use of primary human cell models. The marriage of 3D cell culture, microfluidics and engineering has given rise to a variety of platforms for micro-physiological systems. With rapidly evolving portfolios of the pharmaceutical industry, a high need for new tools to meet the challenges is evident. In particular, methods need to be developed to assess the efficacy and safety of an increasing number of antibodies that have no orthologous target in any non-clinical species. Likewise, a growing number of programs target immune-related pathways that are either not present in animals or show very different regulation compared to humans. While human cell systems are highly desirable for such drug development programs, establishment of cell models that are able to reproduce a physiologically relevant immune response is not trivial and significant investments are needed before these models can be used reducing or even replacing animals. In order to win, technical advancements have been important for these models and sourcing of human cells will be key for the future. Once all these considerations have been brought together, such models will reach their potential to initiate a quantum shift in the development of novel effective and safe drugs.

**Presentation:** Oral

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## Barriers to the implementation of animal-free alternatives and how to overcome them

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The field of alternatives research has accelerated in the past 30 years, largely as a result of legislative pressures on specific sectors to end animal testing and/or use non-animal methods (NAMs). It is widely acknowledged that public pressure has played a significant part in encouraging these developments, particularly in the area of cosmetics and that this has had a positive impact on other sectors.

There are now NAMs for many of the standard animal tests that are typically required to test the safety of new chemicals and drugs. Unfortunately, the corresponding removal of the animal test is still forthcoming. The replacement of an animal test is a laborious and lengthy, scientific and bureaucratic process, from development to validation to adoption of a formal test method to regulatory acceptance which may require adoption in specific legislation before ending with the deletion of the corresponding animal test. Unfortunately, the process is often repeated for each sector in which the NAM is applicable.

There are many reasons why animal testing continues even after the adoption of a NAM. Some of these have little to do with the scientific limitations of the new tests. Human limitations, including bureaucracy, political malaise, and entrenchment in the scientific community are as great, if not greater, barriers to the replacement of animals in testing. This presentation discusses the barriers to the implementation of NAMs and how these might be overcome. Better communication by regulators regarding their acceptance of NAMs supported by stronger enforcement are particularly important. Greater funding to develop replacements for animal tests is still very much needed. Central EU funding for example is only around 35 million Euro per year (0.4% of EU central science funds) and national funding by EU member states is less than 0.04% of their national science funds.

**Presentation:** Oral



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## Screening to assessment: Building confidence in bioactivity points of departure at Health Canada

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Traditional approaches for chemical testing cannot keep pace with current regulatory demands for prioritization and risk assessment. The past decade has seen significant efforts internationally that have provided strong scientific evidence for the utility of emerging technologies and alternatives to traditional testing approaches, or New Approach Methodologies (NAM), to rapidly and effectively identify and assess hazard in support of a risk assessment paradigm shift. A number of Health Canada program areas have initiated efforts to advance the science and application of NAM, including moving toward the use of high-throughput and high content approaches to derive bioactivity-based points of departure (PODs), or POD-NAM, and for application in tiered testing paradigms. The POD-NAM is currently being explored as a protective estimate of traditional PODs to support hazard identification, or when coupled with exposure, to serve as a broad risk-based approach for prioritization and to inform screening level assessments. The case studies presented will demonstrate the utility of POD-NAM to identify priority substances and for further application in a tiered assessment paradigm with a focus on high throughput screening data analysis, *in vivo* toxicogenomics and comparisons with apical endpoints derived from conventional rodent tests (Gannon et al., 2019; Paul Friedman et al., 2020). This presentation will highlight the success to date in advancing the use of new technologies through case studies that illustrate the practical and positive impacts of modernization in decision-making contexts but will also acknowledge the shortcomings associated with *in vitro* approaches. The advancements discussed are the result of strong collaborations between the research and regulatory communities, Internationally and across Health Canada Directorates and Branches demonstrating confidence building through the refinement and implementation of emerging methodologies.

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**Presentation:** Oral

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## Exploration of an animal-free drug development approach for tomorrow's medicine

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A high attrition rate is observed during the expensive clinical phases of drug development, which has been frequently attributed to limitations in the translational aspects of preclinical models (both *in vitro* and *in vivo*). Given the ethical considerations inherent to *in vivo* studies, the goal of this project was to study whether it is possible to integrate *in vitro* and *in silico* modelling technologies in such a way that this will give sufficient information to be able to start initial testing in human volunteers. The current study is a proof-of concept study aimed to identify hurdles that are encountered when attempting to perform animal-free drug development; to pinpoint current technical limitations and to identify potential solutions to overcome these hurdles. Although the developed approach is intended to be applicable to any given disease area, the current study was limited to NASH-associated liver fibrosis as a prototype disease as the number of patients with this disease is increasing rapidly while currently no effective drugs are available. In addition, the study was limited to the efficacy aspect of drug development since there are no harmonized regulatory guidelines to efficacy studies (unlike safety studies) and is therefore more open to innovative approaches. A transition to animal-free drug development requires establishment of how translatable *in vitro* models are to the pathophysiology of the patient population at target. For this, detailed knowledge of disease mechanisms is crucial. This was obtained by integration of *in silico* and *in vitro* modelling technologies, using data mining, text mining, experimental *in vitro* data and systems biology. First results from this approach will be presented

**Presentation:** Oral

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## Promoting transparency in preclinical research: Preregistration of animal studies on an online platform

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Publication bias, selective outcome reporting and risk of other biases occurring limit the validity and reproducibility of animal studies and threaten their translational value (Voelkl et al., 2018). Preregistration is a promising process to reduce these limitations and create transparency. For clinical trials preregistration is standard however, this is not the case for animal study protocols, whilst these form the basis for clinical research. In this initiative we have developed an online platform to preregister animal study protocols (Jansen of Lorkeers et al., 2014) and open to researchers worldwide.

An expert group on preclinical evidence synthesis designed the registration form which consists of 34 fields. Details of the study's hypothesis, design, outcome measures (primary and secondary), measures to reduce bias and sample size rational are asked for. Authors need to indicate whether their study is exploratory or confirmatory. Reference to publication(s) or data repositories can be provided. Protocols can be made publicly directly after submission or after an embargo period. This online platform aims to provide an overview of all executed animal studies, including those that remain unpublished and thus contribute to a reduction of publication bias and unnecessary duplication. It allows reviewers and researchers to access additional information on the study design. Finally, our initiative aims to increase awareness and transparency concerning risk of bias and selective outcome reporting and potentially reduce these biases (Nosek et al., 2018).

We believe all stakeholders involved in animal research should encourage preregistration and call upon researchers, institutes, medical journals, funding bodies, policy and law makers, scientific societies and other parties involved to make prospective registration the standard and mandatory in animal research.

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**Presentation:** Oral

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## Understanding nanomaterial risks in pulmonary infection: Effects of graphene related materials on healthy and *Streptococcus pneumoniae* infected 3D reconstituted human lung cells

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The growing industrial and commercial production of graphene related materials (GRMs) and GRM-based products has led to an increased risk of potential human exposure and raised scientific and public concern about human health and safety. Even though human exposure to GRMs can occur via different pathways, the main exposure route for airborne GRMs is through the lungs, thus placing the respiratory tract at particular risk. Occupational exposure to GRM might occur repeatedly over a prolonged period of time, hence it is crucial to obtain a mechanistic insight into the lung toxicity of long-term, repeated, low dose administration. Another aspect to consider in GRM occupational exposure is that exposed individuals might suffer from respiratory bacterial infections, caused by for instance *Streptococcus pneumoniae* (SP). Therefore, our aim was to investigate the chronic consequences of GRM exposure in both healthy and SP-infected reconstituted primary human bronchial epithelium to assess if GRMs increase the susceptibility to lung infections.

Our findings revealed that long term (up to three weeks), repeated exposures at realistic low GRM concentrations did not induce significant biological responses in healthy cells, as demonstrated by cytotoxicity, membrane integrity and cytokine profiling measurements. However, preliminary results have shown that SP-infections on long-term GRM exposed cultures resulted in impairment of the barrier integrity and an increased bacterial basolateral transmigration, indicating that exposed individuals may be more susceptible to SP bacterial infections. More exper-



iments are conducted to determine whether chronic exposure to GRMs is critical for the invasiveness of lung infections.

**Presentation:** Oral

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## Hands-on experience with the application of NAMs for the registration of petroleum substances under REACH

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Although new approach methodologies (NAM) have progressed the regulatory acceptance of alternative testing methods for relatively simple (lower tier) endpoints and well-defined chemicals, the challenge is in minimizing animal testing for the more complex, higher tier human health endpoints for the risk assessment of complex- and multi-constituent substances such as UVCBs (Unknown or Variable composition, Complex reaction products and Biological materials).

Petroleum substances are a prototypical example of UVCBs. Regulators and industry have a common interest to define a process for (petroleum) UVCBs to ensure that there is no underestimation of hazards and at the same time minimize, or eventually eliminate, the use of animals in toxicology testing to ensure safe use.

Concawe (<http://www.concawe.eu>) has several ongoing research efforts aiming to progress the risk assessment of petroleum UVCBs – focusing on either directly informing human health hazard assessments (e.g., reprotoxicity and carcinogenicity endpoints) as part of a “weight of evidence approach”, and indirectly by informing and underpinning grouping and read-across approaches. Both are realistic short term applications of high content screening tools, and examples on how these are applied on petroleum UVCBs will be presented, with a particular focus on the integration of these and other relevant data types around a multi-year research consortium initiated in 2016 by Concawe: Cat-App (<https://www.concawe.eu/cat-app/>), which is, applying high-content screening data to underpin grouping and read-across under regulatory programs such as REACH (EC, 2006).

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**Presentation:** Oral

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## The interest to adopt a change on TABST & LABST in Brazil

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In Brazil, the Ministry of Agriculture is the responsible for the veterinary vaccines regulations, that includes the Target Animal Batch Safety Test (TABST) and the Laboratory Animals Batch Safety Test (LABST). Because vaccine production has made significant progress through introduction of strict controls over starting materials and development of Good Manufacturing Practice, Quality Assurance and Control and Pharmacovigilance systems, a proper environment is in place in which these safety tests could finally become obsolete. The Ministry of Agriculture is very interested in collaborating with key stakeholders to promote the waiver and – eventually – the deletion of TABST and LABST and its approach and activities are presented by Dr. Marcos Vinicius Santana Leandro, coordinator of veterinary medicines.

**Presentation:** Oral

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## Human hepatic *in vitro* models reveal distinct anti-NASH potencies of PPAR agonists

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**Background:** Non-alcoholic steatohepatitis (NASH) is a highly prevalent, life-threatening, chronic liver disease characterized by hepatic lipid accumulation, inflammation and concomitant fibrosis. Up to date, no anti-NASH drugs have been approved. A major reason is that *in vivo* studies have failed to accurately reproduce human NASH characteristics (Boeckmans et al., 2018).

**Aim:** To evaluate the anti-NASH properties of 8 peroxisome proliferator-activated receptor (PPAR)-agonists (bezafibrate, elafibranor, fenofibrate, lanifibranor, pemafibrate, pioglitazone, rosiglitazone, saroglitazar) using human hepatic *in vitro* models and to develop an *in vitro* scoring system to classify compounds according to their anti-NASH potencies.

**Methods:** Primary human hepatocytes (PHH), HepaRG, HepG2 and skin stem cell-derived hepatic cells (hSKP-HPC)



were triggered with factors that play a role in the onset of NASH. Lipid accumulation, secretion of inflammatory mediators, ATP-content and apoptosis were measured to assess the *in vitro* NASH status. In addition, LX-2 human hepatic stellate cells were exposed to TGF- $\beta$  to induce a pro-fibrotic response. Ultimately, anti-NASH potencies were graded according to an adapted version of the widely employed “non-alcoholic fatty liver disease (NAFLD)-activity score” that is used to evaluate liver biopsies.

**Results:** All evaluated *in vitro* models recapitulated key features of NASH. The parenchymal models showed intracellular lipid accumulation and secretion of inflammatory chemokines, whilst LX-2 cells showed increased pro-fibrotic gene expression. PPAR-agonists attenuated lipid accumulation and inflammatory chemokine secretion in all models, but most stringently in PHH and hSKP-HPC. Additionally, PPAR-agonists reduced pro-fibrotic gene expression in TGF- $\beta$ -stimulated LX-2 cultures. An *in vitro* scoring system based on PHH, hSKP-HPC and LX-2 cultures showed that elafibranor, followed by saroglitazar and pioglitazone induced the strongest anti-NASH effects, corroborating clinical data (Boeckmans et al. 2020).

**Conclusion:** Human-based *in vitro* models can recapitulate several cellular NASH characteristics. Hence, a combination of *in vitro* NASH models can significantly contribute to the testing of anti-NASH properties of drug candidates.

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**Presentation:** Oral

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## Increasing the reliability of preclinical data: Enabling approaches

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Data generated in nonclinical research studies drive decisions with long-term consequences. Concerns about the extent to which these data can be relied upon, initially raised in evidence-based scientific articles, have become part of a worldwide conversation on what is known as the “reproducibility crisis”. In neuroscience research, for example, systematic reviews, meta-analyses and multicenter studies continue to demonstrate that multiple sources of bias and differences in practices can affect

data and conclusions, and ultimately decision-making. While the debate continues to unfold, initiatives aiming to reduce the risks of bias and to increase the reliability of preclinical data are being developed. Reporting guidelines and best practice recommendations have made important contributions, but there is still a need for enabling approaches. A common framework, with comprehensive evidence-based guidelines and practical tools would help scientists ask a scientific question, appropriately design experiments to answer the question, and generate and share data that they and others will be able to rely upon.

**Presentation:** Oral

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## Potential application of new approach methodologies to improve regulatory acceptance of read-across

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The use of existing information from well characterized substances to “read-across” to structurally similar uncharacterized chemicals is a widely used tool to fill data gaps in (eco)toxicological profiles, however there are still limitations in its use and acceptance for regulatory purposes. This approach as an alternative to testing is of particular interest under regulatory frameworks where animal testing should be performed as a last resort, as with the EU REACH Regulation (Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and restriction of Chemicals), and where less experimental data is available (usually the case with high tier human health endpoints). Addressing more complex systemic toxicological endpoints (such as Repeated dose toxicity and Reproductive/Developmental toxicity) with a read-across approach remains a challenge. Common reasons for rejection of a study include lack of data to/data does not support predictions based on toxicokinetics, substance identity, chemical similarity/impact of the difference in moieties between target and source substances, and toxicological similarity/observable trend in the toxicological properties of target and source substances. In this context, NAMs (New Approach Methodologies) could present a way forward to bridge identified gaps and increase the robustness of a proposed hypothesis. An analysis of issues identified in read-across studies submitted under the EU REACH Regulation is presented, together with an overview of how NAMs could be used to support read-across studies and potentially improve their acceptance within a regulatory framework.

**Presentation:** Oral



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## Toxicity pathway- and mechanism-based risk assessment of chemical-induced mitochondrial toxicity

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A pathway and mechanism-based toxicity testing strategy for chemical safety assessment has been increasingly playing a pivotal role in the Next Generation Risk Assessment (US EPA 2014) without using animals. Many studies suggest that chemical-induced mitochondrial toxicity could potentially result in multiple adverse effects in human. The present study was designed to establish a tiered toxicity pathway and mechanism-based approach to assessing chemical-induced mitochondrial toxicity. A group of chemicals labeled as cardiac and/or hepatic mitochondrial toxicants were studied for their cytotoxicity and mitochondrial damage in different human-originated cell models, including human induced pluripotent stem cell (hiPSC)-derived cells and three-dimensional (3D) cultured HepaRG cells. To identify relevant biochemical (toxicity) pathways mediating mitochondrial injury biomarkers, a group of *in vitro* assays combined with computational modelling were used to evaluate some adverse mitochondrial effects at different biological key events. Two pathways (PGC-1 $\alpha$ /Nrf2) were recognized as toxicity pathways regulating mitochondrial oxidative stress and biogenesis which are critically involved in mitochondrial dysfunctions (Yuan et al., 2016). Perturbation of these pathways was particularly important and considered to be linked to the adaptive/adverse effects in cells. Based on the *in vitro* data and *in silico* simulation, an integrative analysis was performed to derive toxicological point of departure (POD) for cell death, oxidative stress, mitochondrial dysfunction, as biological pathway-altered doses (BPAD). Moreover, to extrapolate *in vitro* data to *in vivo* situations, physiologically based kinetic (PBK) models were also developed to predict the external dose levels. Our results indicate that the tiered toxicity pathway and mechanism-based strategy paves the way for assessing chemical-induced mitochondrial toxicity in humans without the need for animal testing.

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**Presentation:** Oral

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## Probabilistic modelling of an adverse outcome pathway network for developmental neurotoxicity

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Over the past decade, there has been an increased recognition of the need for new approaches for the hazard identification and screening of compounds with potential developmental neurotoxicity (DNT) (Bal-Price et al., 2015). Moreover, as DNT is a highly complex process brought about by many chemicals acting via multiple modes of action, identification of common key events within an adverse outcome pathway (AOP) network for human neurotoxicity is essential for the better depiction of chemical exposure and associated apical endpoints (Spinu et al., 2019). Importantly, data-driven models that combine *in vitro* and *in silico* information have the potential to better predict toxicity compared with single methods, thereby enhancing the safety assessment. As such, this study aimed to investigate the applicability of the Bayesian approach to quantify a simplified AOP network for developmental neurotoxicity. Empirical data were used to predict the probability of a chemical inducing DNT associated key events. The model was based on a set of 97 compounds, comprising pharmaceuticals, industrial chemicals and pesticides, of which 73 had *in vivo* DNT evidence. The Bayesian model was informed by details such as physicochemical properties, e.g., logarithm of the octanol-water partition coefficient (log P), blood-brain-barrier permeability, interaction with P-glycoprotein, as well as data from *in vitro* assays that utilised human neuroprogenitor cells and rat primary cortical cells. The analysis was implemented in PyMC3, an open-source Python package for Bayesian statistical modelling. The model incorporated diverse types of data and quantified all sources of uncertainty demonstrating the advantages of Bayesian machine learning methods and their potential application to quantify an AOP network for hazard identification. Acknowledgement: funding from the EU in3 Marie Skłodowska-Curie



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## A human hepatocyte-like cell based *in vitro* model for hepatic insulin-driven *de novo* lipogenesis

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Steatosis, marked by increased intra hepatic triglyceride accumulation, is a hallmark of non-alcoholic fatty liver disease (NAFLD) and precedes the progression to non-alcoholic steatohepatitis (NASH) and liver fibrosis. Hepatic *de novo* lipogenesis (DNL), activated by glucose and insulin, is a major pathway in the development of steatosis and contributes to 38% of the intrahepatic triglyceride-palmitate content in NAFLD patients (Smith et al., 2020). Recent studies in both animal models and NAFLD patients demonstrated that a reduction in steatosis is associated with an improvement of NASH and hepatic fibrosis, indicating the therapeutic potential of drugs acting on hepatic steatosis (Harrison et al., 2019; Gapp et al., 2020). Currently there is a lack of human *in vitro* hepatocyte models that can support the identification of novel drugs inhibiting hepatic DNL. None of the existing models are described to be sensitive for insulin driven DNL, while the available rodent hepatocyte models (*ex vivo* or precision-cut liver slices) have insufficient throughput for effective drug discovery. In collaboration with the lab of In Vitro Toxicology and Dermato-Cosmetology of the Vrije Universiteit Brussel (VUB), we identified that the human hepatocyte-like cells (HLCs) (Natale et al., 2018; Boeckmans et al., 2019), derived from skin precursor cells (hSKP), are uniquely sensitive to insulin driven DNL, shown by both gene expression and lipid accumulation readouts. We demonstrated that the sensitive HLCs showed an increased SREBP-1C expression, a key transcription factor for DNL, upon insulin stimulation. Moreover, inhi-

tion and activation of the DNL pathway could be demonstrated using reference inhibitors (ACCi and AKTi) and activators (LXRa). After miniaturization of the lipid accumulation assay to a 384-well plate format, a library of publicly available mode-of-action chemical substances was screened to validate the relevance of the model and to identify novel targets involved in the DNL.

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## Bottom-up physiologically based toxicokinetic modelling of perfluorooctanoic acid

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Perfluorooctanoic acid (PFOA) is a common industrial chemical found in the blood of over 98% of the populace. Unlike most compounds, PFOA has an extremely long half-life of 2.7 years, bioaccumulating in the body to cause adverse effects including dyslipidemia and developmental toxicity. Currently, a limited understanding of PFOA toxicokinetics (TK) hampers an assessment of its safety. Animal data indicate that the half-life of PFOA is 4-6 days in male rats, which is vastly different from that in humans. These divergent findings are attributed to species differences in transporter expression and suggest that extrapolation of animal-derived PFOA TK data may be unreliable. PFOA is metabolically inert and highly ionized at physiological pH, thus its disposition is likely driven by transport processes. Bottom-up physiologically based toxicokinetic (PBTK) modelling was used to predict the transporter-dependent disposition of PFOA and rationalize the mechanisms underpinning its long half-life. We conducted *in vitro* uptake transporter assays for 8 transporters (OAT-P1B1, 1B3, 2B1; OAT 1, 3, 4; URAT1 and NTCP) representing



uptake into the liver and kidney. Kinetic parameters obtained ( $J_{max}$ ,  $K_m$ ) were incorporated into the Simcyp<sup>®</sup> Simulator. Our simulations accurately reproduced the long half-life of PFOA (39 days), and simulated plasma concentrations, area under the curve, volume of distribution and clearance values were within two-fold of observed clinical data (Elcombe et al. US 2013/0029928) for both single and multiple exposures. Our mechanistic model suggests the long half-life of PFOA is primarily attributed to poor renal clearance rather than biliary excretion. The accurate prediction of the TK profile of PFOA opens new possibilities to predict TK parameters for other per- and polyfluoroalkyl substances, including tissue concentrations and extrapolation to other populations such as the young, elderly and pregnant.

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## Validation of a bottom-up PBPK model prediction of hepatic concentrations of rosuvastatin

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Understanding the unbound drug concentrations within various tissues of the body is necessary to fully appreciate its pharmacodynamic and toxicodynamics (PD/TD). Recent advances in physiologically based pharmacokinetic (PBPK) modelling, *in vitro*-to-*in vivo* extrapolation methodologies and permeability-limited pharmacokinetic models have improved the predictions of tissue drug concentrations without the need for animal data. However, suitable *in vivo* tissue concentrations to validate the model prediction accuracy are often lacking. In 2019, a positron emission tomography (PET) imaging study quantifying the concentrations of [<sup>11</sup>C]rosuvastatin (RSV) in the liver was published (Billington et al., 2019). This afforded a unique opportunity to validate the performance of our previously published bottom-up RSV PBPK model in predicting transporter-dependent, hepatic concentrations (Chan et al., 2019). We utilized the Simcyp<sup>®</sup> simulator and the permeability-limited liver (PerL) model, coupled with extensive *in vitro* transporter kinetics and proteomics-based scaling factors in our RSV model. However, the PerL model does not adequately describe the physiology of the liver as it fails to account for the presence of RSV in the bile canaliculi that was

detected in the PET imaging scan. Thus, to recapitulate the hepatic concentrations in the initial phase of RSV distribution, we excluded the biliary excretion kinetics from the model. After a 0.91 µg RSV intravenous dose, simulated RSV area under the plasma concentration-time curve (AUC<sub>0-30min</sub>) and maximum plasma concentration ( $C_{max}$ ) were predicted within 1.5-fold of the observed data. Crucially, for hepatic RSV concentrations, the model recapitulated the observed hepatic concentrations accurately. Simulated area under the liver concentration-time curve (AUC<sub>0-30min,liver</sub>) and maximum liver concentration ( $C_{max,liver}$ ) were predicted within 1.5-fold of the observed data. In summary, we validated the ability of fully bottom-up PBPK modelling to predict hepatic RSV concentrations. We demonstrate the robustness of the bottom-up PBPK approach to predict the tissue time-course of xenobiotics, which in turn can be correlated with toxicodynamic readouts for risk assessment.

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## The zebrafish embryo as alternative model for acute and chronic fish toxicity – inclusion of additional endpoints to replace fish toxicity tests in the comparative assessment with *Daphnia* and algae

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Information on acute and chronic toxicity is required for environmental hazard classification and labelling of chemicals. This information is typically obtained using a combination of standard tests with fish, *Daphnia* and algae. As indicated by Rawlings et al. (2019) replacing the acute fish toxicity test by the fish embryo acute toxicity test (FET) has a minimal impact on the final classification and labelling of acute toxicity. Thus, the FET represents a sensitive alternative model. We examined whether a similar rela-



tion can be obtained for chronic fish toxicity (based on the FELS – fish early life stage – test). Therefore, a database of FELS toxicity data for 223 compounds with corresponding *Daphnia* and algae chronic toxicity was compiled (Teixido et al. 2020). 9.5% of the investigated compounds showed a  $\geq 10$ -fold higher sensitivity in the FELS test. Some of these compounds are considered as endocrine disrupting or exhibit different specific mechanisms of action. By targeting these mechanisms with additional endpoints, the predictive capacity of the FET may be further improved: (i) For acute toxicity, it had already been shown that correlation of FET and acute fish toxicity test may be improved by including additional sublethal endpoints, particularly the assessment of behavior (embryonic movements). Some of these sublethal endpoints such as the touch-evoked response can be added to the standard FET (OECD 236) with minimal additional workload and no need for specific equipment. (ii) For chronic toxicity, adding the assessment of morphology and endpoints for endocrine disruption (e.g., via reporter gene analysis) could improve the predictive capacity of the FET. Hence, by combining the FET with additional endpoints a similar hazard prediction is likely to be achieved as would be obtained with established animal tests.

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## Data driven computational modelling to support safety assessment of cosmetics ingredients

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The safety assessment of cosmetic ingredients has undergone a radical change in the past decade due to legislative requirements. This has resulted in the need for novel thinking and implementation of new techniques and innovative approaches. Computational approaches are at the heart of many of the new techniques for the safety assessment of cosmetics ingredients. Recent years have seen an increase in the amount of data that can support these approaches with many resources now being available (Pawar et al., 2019). For instance, an inventory of cosmetic ingredients is available that provides an insight into chemical space (Yang et al.,

2017). With the chemical space mapped it is possible to see where we have good numbers of *in vitro* and *in vivo* data to support new approaches. The data repositories have supported the development of (internal) threshold of toxicological concern (iTTC) values that are more appropriate to the cosmetic space (Yang et al., 2017; Ellison et al., 2019). There has also been a great improvement in the development of approaches for read across and other *in silico* models including quantitative structure-activity relationships (QSARs) and application of machine learning. The cosmetics industry has taken a lead in the development of workflows and frameworks to fill data gaps when no suitable data, or read across information, is available (Dent et al., 2018). For instance, ab initio workflows combine various sources of information to make safety decisions based on possible exposure and hazard information. These new data driven computational approaches are being developed in a transparent manner with the aim of achieving regulatory acceptance and greater uptake (Rogiers et al., 2020).

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## Improvement of *in silico* models for toxicity prediction by identifying, characterising and reducing uncertainties

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*In silico* models for toxicity prediction, such as quantitative structure-activity relationships (QSARs) and read-across, are used to predict the toxicity, fate and properties of chemicals and have found great utility for regulatory applications. The key to the practical application of predictions is their acceptability for regulatory purposes, this has historically been guided by the



OECD Principles for the Validation of QSARs as well as copious guidance, for example from OECD and ECHA. To improve this process, uncertainties, variability and areas of bias have been defined for read-across (Schultz et al., 2019) and QSARs (Cronin et al., 2019). For instance, 49 assessment criteria have been provided for QSARs. The aim of the present study was to utilise these uncertainty schemes for QSARs for toxicity to demonstrate their applicability (both of the schemes and QSARs), review their use and attempt initial quantification. The schemes to assess the uncertainty, variability and areas of bias of QSARs were applied to published QSARs. Each QSAR was evaluated according to the questions defined by the schemes. The evaluation of the QSAR models found that they were generally well described and presented, although for some models provenance of the data was uncertain. Areas where QSARs could be improved included the mechanistic evaluation and justification of the models and the definition of the compound / data set collection. The assessment criteria were found to be easy to apply and gave confidence to predictions from the QSAR models.

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## The windy road to the use of non-animal approaches for regulations of chemicals

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In Europe, the use of animals to test food and drinks has become a public concern and an issue for the food industry and a lot of debate has surrounded the use of animal studies in food safety, especially regarding identifying when they are mandatory or not. Animal testing is still predominant in the food ingredient industry, despite the recommendation to use alternative methods and efforts to foster development and acceptance of alternative methods. Regulators and consumers mandate the use of animal-free approaches for the risk assessment of food ingredients. But no testing bans exist such as for the cosmetics industry or regulations such as REACH for the chemical industry, despite increased attention from the media and public opinion. For instance, the use of alternative methods must fulfil certain requirements (e.g., reproducibility, precision and practical applicability as well as predictivity and relevance) to be used in the chemical industry. But often these requirements refer to an animal method

or the regulation itself was designed with animal studies. The development of alternatives strategies to animal testing offers new opportunities for assuring food safety (e.g., alternative assessment methods like TTC, read-across, or computational methods are gaining importance in the food industries), but is still needed to address the majority of toxicological endpoints, which are currently addressed by animal methods. While a wealth of new methods is being developed, the regulatory validation and use of these methods is lagging behind.

**Presentation:** Oral

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## Asia is a ripe place for alternatives to animal testing: Status and potential in India

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The low translational efficiency of animal models has been well documented globally. The situation is similarly dire for India, where drug development has resulted in more than 200 compounds that entered preclinical and clinical stages; however, none reached the global markets in the last two decades. Thus, even in the Indian context there is an urgent need to develop more relevant and efficient models for research and drug development.

While mice (69%) constitute a major animal model in India, computational studies (24%) come a close second. These resources could be further harnessed to create predictive models and reduce animal experimentation. In the last five years, almost 30 labs spanning the country have started working in the areas of organoids and organ-on-chip.

In the past decade, India has also introduced several regulatory changes to reduce animal usage in experimentation and education, including ban on the “Draize test” and import of animal-tested cosmetics. In 2018, Central Insecticides Board Registration Committee (CIBRC), Ministry of Agriculture revised its regulation to recognize human cell-based alternative testing methods.

To promote the area of human-relevant research, the Department of Biotechnology (DBT, Ministry of Science and Technology, India) recognized “Stem Cells and Regenerative Medicine” as one of the thrust areas for research and development. However, only 0.2% of its total funding was allotted to alternatives to animal research in the last four years.

Thus, there is a need to increase awareness towards the value of investing and refining the non-animal methods.

With the vast proportion of computational researchers, a growing number of experimentalists working on alternative to animal experiments, and regulators beginning to understand its



potential, India has the potential to become a global front in the research, development, and application of human-relevant research.

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## Brazil is on: Animal testing ban and available OECD TG in Brazil

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In 2019, Brazil joined the growing list of countries banning animal experiments. In 2014, the National Council for Animal Experimentation (Concea) Normative Resolution No 18 (RN18) made the use of 17 Alternative Methods mandatory, establishing five years deadline for the public and private sector to adjust to this new reality. Among the endpoints, skin irritation and corrosion tests required full availability, as this endpoint is broadly demanded several industries and different types of products. Since 2012, The National Institute of Metrology, Quality and Technology (Inmetro) has been one of three Central Laboratories of the National Network of Alternative Methods (Renama), established to promote the implementation, dissemination and validation of Alternative Methods to Animal Experimentation.

The PRéMASUR (MERCOSUR's Regional Platform for Alternative Methods for Animal Experimentation) National initiative had organized by far 19 training courses focusing on *in vitro* methodologies leading the training approximately 200 professionals from Brazil and Mercosur member countries. In this context, training and dissemination efforts are crucial to prepare and anticipate the needs in terms of demand, but also to create an efficient network to widely deploy these methods throughout the country. It is worth mentioning that the production in Brazil of the *in vitro* reconstituted human epidermis by Episkin's subsidiary in Brazil was a milestone, making available an emblematic model of replacement alternative for research, training and regulatory purposes.

In 2014 there was no GLP laboratory in Brazil for RN18 CONCEA *in vitro* methodologies. Nowadays, there is currently at least one test facility for the OECD TG 431, 439, 437, 428, 487 methodologies required by RN18 CONCEA, allowing Brazil to adhere to 3Rs (reduction, refinement and replacement) principles and the sequential access to markets abroad. The partnership between the public and private sectors was crucial to achieving this success.

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## Enhancing preclinical predictions for neurodegenerative diseases using brain-on-chip models

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Drug development for neurodegenerative diseases is hampered by the limitations of current preclinical models, in large part due to physiological differences between animal models and humans (Finkbeiner, 2010). While conventional cell culture cannot substitute animal models, promising advances are being made via both technological and biological avenues.

Microfluidic platforms, here specifically Brain-on-Chip (BoC) platforms, allow for integration of micro- and nanoscale technology for (real-time) analysis, robust and controllable cellular microenvironments, and increased assay throughput at lower volumes.

From a biological perspective, human induced pluripotent stem cells (hiPSCs) can be utilized to generate disease- and human-specific neuronal cell cultures or organoids (Bordoni et al., 2018).

A proof of concept is required to showcase the feasibility and potential of BoC-enabling technology in combination with hiPSCs to translate scientific findings into relevant preclinical models for drug discovery towards neurodegenerative diseases. Innoser focuses on enhancing the translational power of preclinical disease models by pioneering *in vitro* brain models based on hiPSCs in collaboration with both academic and commercial partners for Alzheimer's Disease (AD), Amyotrophic Lateral Sclerosis (ALS) and Parkinson's Disease (PD).

Specifically, the MINDMAP consortium (Eurostars project E113501) aims to deliver an AD BoC platform with dynamic optical imaging of 3D hiPSCs-derived neuronal cultures, supported by AI-driven software to further enhance predictability in drug and disease development.



The Nano+ consortium (Health Holland project LSHM19006) aims to integrate nanotopography with electrophysiological measurements on 3D hiPSCs-derived motor cortex neurons for ALS.

Last, through our partnership with OrganoTherapeutics we make midbrain organoids (Monzel, 2017) available for screening of new drugs in preclinical PD research.

These collaborations allow us to build a commercially viable BoC model ecosystem. With these projects, we ultimately strive towards a future of preclinical models with superior clinically translational relevance for neurodegenerative diseases with less use of animal models.

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## Endothelium response to iron nanomedicine in static versus dynamic *in vitro* vascular model

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Nanomedicines are promising therapeutic compounds allowing the development of new treatment approaches with reduced side effects. However, important factors affecting the behavior of nanoparticles *in vivo* cannot be simulated in conventional static *in vitro* models (Praschberger et al., 2015). Dynamic cell culture, where cells are cultivated in the presence of shear stress has the potential to bridge this gap by mimicking critical features of physiological conditions. Intravenous iron-carbohydrate nanoparticles are becoming a dominant treatment for the correction of acute iron deficiency unresponsive to oral iron supplements (Cançado et al., 2011). Compared to the data available from clinical studies, little is known about the interaction of iron-carbohydrate nanoparticles with endothelial cells (ECs), which are the first contact cells for circulating iron nanoparticles following intravenous administration (Zou et al., 2017). Our approach implements and compares a microchannel-based dynamic human endothelium model to the static culture to assess capability to predict differences in EC response after exposure to intravenous iron sucrose (IS). Differences in cellular uptake were confirmed using three approaches, including transmission electron microscopy, flow cytometry, and Prussian blue staining assay. In-

crease in the expression of E-selectin, an adhesion molecule expressed only on endothelial cells activated by inflammatory responses, was also noticeable in ECs exposed to IS under static condition. Our results showed that cytotoxicity to ECs caused by IS is significantly lower under dynamic condition compared to static culture. Here, we demonstrated that inclusion of dynamic flow and biological fluids are positive steps towards generating nanoparticles evaluation in a relevant *in vitro* model. In future studies, we will seek to further develop the complexity of our dynamic human endothelium system by incorporating phagocytic cells, such as macrophages to achieve a powerful platform for the safety screening of parenteral nanomedicines with high resemblance to *in vivo* models.

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**Presentation:** Oral

808

## Establishing scientific credibility of NAMs. To what extent is transparency, interpretability and explainability necessary?

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Regulatory toxicology is undergoing a paradigm shift with the development and implementation of new approach methodologies (NAMs) in hazard/risk assessment. NAMs include various non-animal approaches for data acquisition and interpretation, such as organ-on-chip and other complex test systems, high content analysis using OMICS or imaging technologies, robotic platforms for high throughput data acquisition, and use of artificial intelligence for data integration and interpretation.

The main goal in this shift of paradigm is to move from the observation and interpretation of apical toxicology effects in animals towards the use of more human- and physiologically relevant *in vitro* and/or *in silico* systems underpinned by mechanistic understanding of toxicological pathways and adversity.

However, many of these new technologies have components protected by intellectual property or confidential business information, which may hinder a knowledge-based assessment of their relevance. In addition, even where information is accessible, the complexity of the technology (e.g., artificial intelligence/



machine learning algorithms) and/or the lack of understanding of biological systems can quickly become limiting factors for such an assessment. This, in turn, can lead to lack of transparency and to purely data-driven demonstration of relevance by comparing to reference animal data. In an era where more emphasis is being given to biological understanding and explainability in the regulatory communities, the increased complexity and need for protection of investment in the development of new technologies are making method developers favor data in detriment of knowledge to demonstrate relevance and establish the credibility of NAMs. However, the lack of transparency may block regulatory acceptance and use, and a purely data-driven approach to establish credibility will continue to suffer from the increasingly evident lack of reliability and relevance of reference animal data to predict human effects.

This talk discusses the current challenges in the validation and regulatory acceptance of NAMs and strategies to address them.

**Presentation:** Oral

809

## Exploiting the use of iPSC-derived renal proximal tubular like cells to investigate megalin mediated aminoglycosides toxicity

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Exposure to environmental pollution and certain pharmaceuticals can adversely affect the kidney and contribute to acceleration of chronic renal disease which is a heavy burden on health care systems worldwide. While human renal cell lines, such as the RPTEC/TERT1 cells are useful tools to study nephrotoxicity, they represent only one genetic background and may have acquired and lost some typical characterizations in culture. One such example is the absence of megalin (LRP2) expression in most of the existing 2D *in vitro* proximal tubular models. iPSC derived models are promising new tools that may be able to overcome some of these limitations. This study investigates the utility of a newly developed iPSC protocol for deriving proximal tubule-like cells (PTL) and the ability of this model to study megalin-mediated aminoglycoside toxicity. Undifferentiated iPSCs were differentiated into PTL and temporal alterations of the cells were characterized by assessing the expression of pluripotency markers, renal developmental markers and maturation markers via immunofluorescence and west-

ern blot analysis. Differentiated PTL cells exhibited a polarized phenotype, barrier formation, and expression of the functionally active proximal tubule specific marker, megalin. To compare the effects of megalin-facilitated aminoglycosides uptake on different proximal tubular models, iPSC derived PTL, human primary proximal tubular cells, RPTEC/TERT1, and HK2 were exposed to aminoglycosides (gentamicin and tobramycin) at the concentration of 12, 250, and 450 µg/ml. Transcriptomic alterations were quantified using the TempO-Seq assay (BioClavis, Ltd). iPSC derived PTL and primary proximal tubular cells showed similar responses with increased interferon signaling and anti-viral response in both gentamicin and tobramycin exposure. On the other hand, RPTEC/TERT1 and HK2 cells did not show any predominant pathway activation. In conclusion, iPSC derived PTL represents an improved human-relevant model for nephrotoxicity and could provide an opportunity to study the effects of compounds that are taken up through megalin-facilitated endocytosis.

**Presentation:** Oral

810

## Stakeholder collaboration to implement regulatory and policy change for drug development

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Efforts around the world aim to replace animal testing by supporting innovative science that does not use animals. Less attention has been given to policies that currently require or favor animal testing over nonanimal approaches. The Nonclinical Innovation and Patient Safety Initiative (NIPSI), a growing group of professionals from federal agencies, the private sector and patient, health and research organizations, was formed to increase the human relevance of nonclinical drug testing. (Baker et al., 2019). NIPSI has begun addressing scientific, educational and policy opportunities to improve drug testing for humans and reduce animal testing. While many NIPSI efforts so far have focused on North America, NIPSI is expanding to include other jurisdictions, such as the European Union. This presentation will provide recommendations to change policies that favor animal studies at a time when industry and regulators recognize the need to integrate more predictive nonclinical approaches.

### Reference

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**Presentation:** Oral



812

## Law and order: Policies and legislation (or lack thereof) for the 3Rs in research

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Increasingly, toxicology and regulatory testing practices include stated or conceptual commitments to reducing and replacing animal studies. These fields are governed by country-specific legislative mandates and defined endpoints and standards driven by internationally harmonized policies (e.g., ICH, OECD) that are adopted nationally through regulatory agencies. In contrast, the use of animals in hypothesis driven biomedical research is not governed by nationally, regionally, or internationally harmonized guidelines that lead the field towards more studies utilizing approaches that incorporate human cells, tissues and processes. Further, direction of research spending tends to follow a diffuse, de-centralized format that discourages centralized planning and prioritization. An absence of any legal requirement to reduce or replace animals used in research or testing further stifles any motivation to make progress. This must change, given the lack of understanding of human disease mechanisms, the dismal success rate of nonclinical studies, and evolving societal ethics. This presentation will highlight differences between European Union and North American requirements to find and use human biology-based methods in biomedical research, as well as policy changes in multiple sectors that may encourage use of nonanimal methods that have the potential to benefit biomedical research by streamlining our understanding of human biology in health and disease.

**Presentation:** Oral

813

## Implementation of the 5Rs (Replacement, Reduction, Refinement, Responsibility and Respect) in laboratory animal science education & training courses in the University of Cape Town, South Africa

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It is essential that all persons involved in the care and use of animals for scientific purposes undergo education and training in order to ensure optimal animal welfare, high standards of animal care and reliable scientific outcomes. The University of Cape Town has developed and implemented education and training courses, including (a) the first FELASA-accredited certification course in Africa for mice and rats for EU functions A & C; (b) an apprenticeship program for Laboratory Animal Technologists; as well as (c) competence-based education and training courses for scientists to perform regulated procedures on animals. Additional short courses in Laboratory Animal Science and Ethics are under development and will include an Advanced Course in Laboratory Animal Science & Ethics as well as competency-based practical training in advanced veterinary procedures.

A core objective of these courses is to impart the principles of the 5 R's and to convey the vital importance of a respectful, responsible, caring and compassionate attitude towards animals. Training systems and processes are designed and implemented to replace the use of live animals wherever possible, to refine education and training methods and to reduce the number of animals used. Methods include the use of ethically sourced cadavers for practical training in restraint and procedures, the reuse of training animals with consideration of cumulative harms, the use of interactive videos and tutorials, reward based positive reinforcement training of rodents, the use of mannequins during training and the employment of non-aversive handling methods. Here we will illustrate the practical implementation of the 5R's principles of replacement, reduction, refinement, respect and responsibility, as part of a Laboratory Animal Science education and training program at the University of Cape Town.

**Presentation:** Oral



815

## A five-category framework for implementing culture of care

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The European Federation of Pharmaceutical Industries and Associations (EFPIA) Research and Animal Welfare (RAW) group members reflected on the concept of a Culture of Care in relation to animal care and use and on differences in its understanding and application across European Pharmaceutical companies (Robinson et al., 2019). The term “Culture of Care” is used across different regions and organisations but rarely with any defined indicators to support working practice.

EFPIA’s RAW group have developed a framework to help organizations identify gaps or potential areas for improvement in support of a positive Culture of Care.

The framework is a tool that identifies five areas of focus for Culture of Care: company values; strategic approach at establishment level; implementation structures; staff support; animal care and procedures. The framework is intended as an aid for continuous improvement, highlighting where indicators of good practice are present. We expect it to provide points of reflection and ideas for those looking to implement Culture of Care in a structured way, while facilitating a professional and strategic approach. To prevent it supporting a “tick-box” exercise, the framework must not be used as an auditing tool, but as a starting point for consideration and discussion about how care manifests within the context and constraints of individual establishments.

Within AstraZeneca we used the Framework to assess culture of care across our sites to enable us to share successful approaches and identify areas for improvement globally.

The presentation will illustrate some examples of successes and areas for further improvement

### Reference

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**Presentation:** Oral

817

## Updating pain recognition and management approaches in laboratory mice and rats

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Pain can be challenging to recognize and treat in laboratory rodents and pain management remains an important ethical consideration for those working with rodents in science. Additionally, animals in pain may not have normal physiologic function or demonstrate normal behaviors, decreasing the reliability of studies in which these animals are used. The International Association for the Study of Pain (IASP) has designated 2020 as the Global Year of Pain Prevention providing an opportunity to focus on pain detection and mitigation strategies for rats and mice. Newer pain assessment techniques, such as facial grimace scoring, burrowing, and nest-building evaluate changes in spontaneous behaviors or animals in their home environments and may prove more useful than traditional evoked response reflex testing. Similarly, an evidence-based review of clinical pain management in rodents suggests that updated recommendations are needed for effective pain mitigation. Careful planning based on study design, knowledge of pharmacokinetics and mechanisms of action of analgesic agents, regular observation of animals for individual differences, and ensuring an institutional culture that recognizes the sentience of laboratory mice and rats is needed for effective clinical management of pain.

**Presentation:** Oral

819

## Practical challenges and considerations in refining euthanasia methods in laboratory animal research in rodents – A pharmaceutical industry case study

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A commonly used euthanasia method for laboratory rodents involves asphyxiation in a rising concentration of carbon dioxide. It offers a cheap and relatively simple method amenable to large



studies; although there is a wealth of literature highlighting welfare issues with this method (Hawkins et al., 2016). For many years this was the method of choice for immune compromised mice used in the oncology research group within AstraZeneca. Many of the oncology research projects build on previous experience within a target or disease area or within an area of synthetic chemistry meaning there is a general conservative approach to change, and this often focussed on the potential impact change can have on the science endpoints of interest. The talk will discuss challenges in identifying and then moving to potentially more welfare friendly euthanasia methods within our Establishment and how working together with the research scientists we have been able to develop an evidence-based decision tree that allowed us to move away from the use of carbon dioxide.

#### Reference

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**Presentation:** Oral

820

## Gold doesn't rust: The failing standard of the animal model

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The animal model has been considered the standard for scientific research and testing for centuries. Animal use has continued in spite of its long history of poor predictivity and relevance for humans as well as its inherent animal suffering. Emerging technologies promise to revolutionize the field of biomedical science by rejecting the failing animal model in lieu of human-based *in-vitro* sciences and other new methods. Can these new technologies break into mainstream understanding and acceptance, or will they be blocked by the sector of the scientific community deeply rooted in and committed to the animal model? The film includes scientific opinion, examples of current and emerging technologies, a brief look at areas of animal use, and excerpts of media coverage. It challenges the long-held rhetoric that the animal model is the “gold standard” for research and testing.

The American Fund for Alternatives to Animal Research (AFAAR) and We Animals Media present this short film as an outreach tool. Its goal is to enhance discussion, inform the public of the current state of alternatives and inspire promise for future advancements that will one day end animal use and replace it with better and fully humane science.

**Presentation:** Oral

821

## The human gut organoids, a promising model to study enterovirus infection and disease pathogenesis

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**Introduction:** Enteroviruses (EVs) are a major source of human infections worldwide, with a broad spectrum of disease symptoms, from diarrhea and skin rash to more severe disease like meningitis and paralysis. Elucidating EV pathogenesis has been limited by the lack of suitable models that faithfully mirror normal human physiology and pathophysiology. Organoids are stem cell-derived *in vitro* 3D organ models and an excellent system that has potential for studying on EV-host interaction, virus evolution, and antiviral compound testing on a human system.

**Methods:** The 3D fetal gut organoids are an “inside out” representation of human physiology with the basal side on the outside facing the environment and the apical side facing the inwards. During culture, the proximal and distal organoids are “opened up” and cultured as a monolayer on transwell inserts to establish viral infection. The monolayers were apically exposed to enterovirus A71 (EV-A71) and subsequent viral replication was assessed by quantifying the production of viral RNA and virus replication at several time points over a course of six days.

**Results:** Using the monolayer transwell system we show that EV-A71 infects the epithelium monolayers from the apical surface. We will present data on infection of the monolayer model with EV-A71, cell tropism of the virus, and monolayer permeability after infection.

**Conclusion:** The human fetal gut derived intestinal organoid model is a powerful model for studying enterovirus infection and related disease pathogenesis. Continued development of the organoids cultures by including components of the normal host tissue microenvironment such as immune cells and blood vessels, will facilitate and simplify studies on human viral pathogenesis, and improve the development of platforms for pre-clinical evaluation of vaccines, antivirals and therapeutics.

**Presentation:** Oral

822

## A comparison of training standards amongst international colleges of laboratory animal medicine

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To assist new colleges of laboratory animal medicine to develop and define quality standards for training and examination of candidates and to help promote intercollege recognition of credentials of Diplomates between the American, European, Japanese and Korean Colleges of Laboratory Animal Medicine (ACLAM, ECLAM, JCLAM, and KCLAM), the International Association of Colleges of Laboratory Animal Medicine (IACLAM) conducted an in-depth review and comparison of oversight, training, credential, and examination standards for each college. Specifically, this included a review of: national or regional college support structures, the college Constitution and Bylaws, a detailed description of qualifying mechanisms for candidates wishing to sit the certification examination, training program criteria, the process for credentialing candidates for examination, the mechanism for examination development, evaluation, and quality assurance, as well as comparing the detailed role delineation documents (RDD) or task analyses for newly qualified Diplomates. While a number of differences were found in processes, there was good general harmonization in approaches to training program duration, qualifications of candidates and credentialing processes, RDDs between the colleges. Areas requiring more detailed review include harmonization of didactic training and certification examination preparation, review, and quality assurance.

**Presentation:** Oral

824

## Potentials and pitfalls of transient fish *in vitro* reporter bioassays

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Regulation drives the demand for animal testing. Agencies and stakeholders are promoting alternatives and implementing the concept of 21<sup>st</sup> century-tox. Future guidelines propose the in-

tegration of effect-based tools and bioassays (WFD re-evaluation). Within aquatic toxicology, coverage of certain MOAs and AOPs is scarce, and most applications are based on mammalian or yeast models, not reflecting realistic exposure scenarios. The use of transient reporter gene assays in organisms of interest (fish) could be a quick and inexpensive solution. However, interference with cellular homeostasis may impact the system beyond the function of the manipulated gene and lead to nonspecific results. We describe how varying vector geometry and different regulatory gene elements on plasmids used for transfection may lead to a large difference in sensitivity.

Zebrafish embryonic fibroblasts and hepatocytes were seeded on 96-well plates, co-transfected with a Nrf2-responsive Firefly luciferase reporter plasmid and 8 different combinations of *Renilla* luciferase normalization vectors. Transfected cells were exposed to increasing concentrations of oxidative stress inducer metazachlor. In response, Nrf2-dependent luciferase induction was recorded via a plate reader. Alternatively, various endpoints of cellular stress (mitochondrial metabolism, membrane stability, protein amount, proliferation) were assessed using non transfected or transiently transfected cells with constructs of increasing sizes.

The results indicate that plasmid geometry and gene-regulatory units have an effect on the potency of the reporter gene assay after co-transfection. Differences in relative induction are a result of the applied normalization vectors, specifically their constitutive promoters and backbones. Viability tests showed that transfection itself increases cellular stress in a construct size-dependent manner. Given that the final signal measured will always be the result of a synergistically acting black-box, precautionary decisions must be taken in plasmid vector design to display weak points and overcome intrinsic faults. In the regulatory context, awareness is important, in order to choose suitable bioassays.

**Presentation:** Oral

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## Test subjects

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Test Subjects is a documentary film made by BAFTA-winning director Alex Lockwood and sponsored by People for the Ethical Treatment of Animals (PETA). The film explores the pressure on aspiring scientists to experiment on animals in order to earn their diplomas. Seen through the eyes of a trio of former doctoral students as they recall their experiences with the expectations of academia, Test Subjects addresses the deeply personal decisions that these scientists made that ultimately changed the course of their lives and careers towards a full humane science. Test Sub-



jects explores the world of the research scientist and what is expected within a professional field that the interviewees argue is not free of the pressure to conform, allows questioning within only a limited framework, and that evidences an inertia that helps maintain the *status quo*, including when specific practices may be contrary to good science. Immediately after the film screening, the interviewees featured in the film will be in attendance to engage with delegates for Q&As.

**Presentation:** Oral

827

## DVM: Training the animal doctor

*Nick Jukes<sup>1</sup> and Olivier Berreville<sup>2</sup>*

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The tools and approaches used within veterinary education and training to gain knowledge and skills are undergoing changes as new technologies are being developed and as ethical and animal welfare concerns are being given greater prominence. This 45-minute beta version documentary film investigates the veterinary curriculum and follows the disciplines, courses and practical classes that veterinary students and trainees will face. The film presents innovative, humane learning tools and approaches that have been developed and implemented by educators to better meet teaching objectives. Interviews with educators, teaching assistants and students illustrate their experience of the alternatives and how they support effective education and training. Demonstrations and practical classes show a range of tools and approaches in use. These include virtual laboratories and virtual reality software for anatomy and physiology education, mannekins and advanced synthetic cadavers for surgery training, client donation programs to provide ethically sourced cadavers, and clinical learning opportunities with animal patients. The film demonstrates that veterinary education and training can be achieved in a fully humane way without harmful animal use, to the benefit of the students, the educators, the animals, and the profession itself.

**Presentation:** Oral

829

## Highlighting modern approaches through education and training

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There are three essential roles that must be maintained for the livelihood of the scientific community: innovators, educators, and regulators. Without these, the rapid advancement of modern approaches (e.g., *in vitro*, *in chemico*, and *in silico* models), adapted or otherwise, would not be possible. Through the role of innovators, scientific literature has documented the development and application of modern predictive tools to protect human health and the environment. Regulators are uniquely tasked with the implementation of safety assessments to protect public health. The work of educators is imperative to evolve the implementation of scientific advancements that challenge historical paradigms, harvest more human-relevant information, and provide holistic environmental approaches. However, the role of an educator is not mutually exclusive; often, educators come from all sectors such as government regulators, industry innovators, and nonprofit organizations influencers. This presentation will overview the rapid development of nonanimal approaches and highlight global educational activities that empower the innovator and regulator to apply modern approaches to research and regulatory assessments. Here, we present an example of this through the Physicians Committee's new training effort – New Approach Methodology Use for Regulatory Acceptance (NURA) – that employs in-person lectures, networking opportunities, online webinars, hands-on demos, and open access online resources. NURA, in addition to other educational and outreach efforts, provides the breadth and depth to allow participation at a variety of levels and endeavors to meet the needs of all towards the goal of implementation of nonanimal methods for regulatory use. The end objective is to enthuse the scientific community and educators to 1) spur innovators, 2) increase communication concerning the necessity of modern approaches within context use, and 3) build confidence of new approaches methodologies.

**Presentation:** Oral



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## Educating future scientists and raising public awareness on animal-free experimentation

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The EU animal protection law, Directive 2010/63/EU, has as its ultimate goal the overhauling of the current research paradigm by fully replacing live animal use. Even though reviews of past animal experiments have revealed that the harms inflicted upon the animals frequently outweigh the benefits (e.g., Pound and Nicol, 2018), animal research proposals are generally authorized by the competent authorities. Any paradigm change away from animal use needs the support of society, but the public has been mostly misinformed by the animal research industry (e.g., Foundation for Biomedical Research; Understanding Animal Research) as well as misled by media stories that frequently tout breakthroughs for human disease based solely on tests carried out on animals (Chakradhar, 2019). There is an urgent need to provide future scientists as well as the public with evidence-based resources about the harms, benefits and limitations of animal experimentation and about ways to replace animals. These observations led me to start offering a university course critically appraising the extensive animal use in science and discussing ways to reduce and replace these animals with more human-relevant methods. The 8-week course at Johns Hopkins University covers the main shortcomings of animal use in science, how to fully apply the 3Rs principles, how to properly conduct literature searches, and how to plan, conduct, analyze and report research studies. The course further teaches how to scrutinize the validity of animal and non-animal models and methods in order to choose the best means for particular research interests (Herrmann, 2019). In addition, we put together a comprehensive book for the interested public in which 51 international experts review current animal use in science and discuss innovative, human-relevant approaches to advance the life sciences and accelerate the paradigm shift towards animal replacement (Herrmann and Jayne, 2019).

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**Presentation:** Oral

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## Developing strategies to increase the use of nonanimal methods to assess food safety

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In recent years, alternatives to animal testing have undergone rapid development in chemical sectors like cosmetics, pesticides, and pharmaceuticals. As a whole, the field of new approach methodologies (NAMs) or alternative approaches – especially for regulatory application – is growing rapidly. Conversely, comparatively little attention has been paid to applying NAMs to food assessments. Food ingredients, food additives, dietary supplements, and food contact materials all undergo extensive toxicology safety testing. Safety assessments can include several studies designed to evaluate genotoxicity, exposures (short-term, sub-chronic, and chronic), carcinogenicity, neurotoxicity, allergenicity, and reproduction and development. If efficacy statements are used, additional testing is commonly conducted. Where available, fit-for-purpose NAMs have reduced the resource strain and provide better predictions than studies that use animals. Implementing strategies to increase NAMs for foods (Blaauboer et al., 2016) is not only timely but necessary. Researchers and method developers seek guidance from regulatory agencies to increase innovation of nonanimal methods. In some cases, such as the US FDA's General Toxicological Principles for the Safety Assessment of Food Ingredients, where Guidelines for Designing and Conducting Toxicity Studies are outlined, regulatory guidance leans heavily towards tests reliance upon animals. Guidance should be modernized to a more flexible framework capable of encouraging the development of modern *in vitro* and *in silico* approaches. This presentation aims to present the state-of-science for nonanimal approaches applicable to food safety assessments, outline areas for improved implementation, and highlight the critical need for scientific advancements such as high throughput systems (Karmaus et al., 2016; Punt et al., 2020) and mechanistic information (Vinken et al., 2020; Kramer et al., 2019). With a wealth of information and rapidly developing innovative techniques to assess single chemicals and complex matrixes, we aspire to apply current advancements with NAMs to foods and food products.

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**Presentation:** Oral

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## Study the effect of cyclosporin A on functionality of endothelial cells differentiated from induced pluripotent stem cells as *in vitro* toxicity model

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**Introduction:** Designing a suitable *in vitro* model of vascular system could lead us understanding the mechanism which chemicals affecting them. Due to 3R principles, we are trying for alternative models to animal testing. Stem cells specially induced pluripotent stem cells (iPSCs) with ability of differentiation to all organs of body could be a good substitute. In this study we differentiated iPSC cells toward endothelial cells using a novel cocktail of small molecules and growth factors as a model for our toxicology assessment. We started our toxicity part using cyclosporin A (CSA) as a potent immunosuppressive agent in pharmacologic studies. Beside the positive effect of CSA, there is emerging evidence showing its effect on inducing long-term vascular dysfunction (Nacev and Liu, 2011; Viillard and Larrivée, 2017) and angiogenesis impairment in patients. We designed this study in order to get deeper insights into effect of CSA on angiogenesis of endothelial cells and finding out the exact mechanisms.

**Materials and Methods:** iPSC cells were derived from two normal donors fully characterized and after proving their pluripotency characteristics were treated for differentiation to endothelial cells. Characterization analysis demonstrated high yield of differentiation. Then we checked the effect of CSA on endothelial cells viability through resazurin assay (Zhao et al., 2015). To understand CSA effect on endothelial cells angiogenesis we modified sprouting assay method. The progress of CSA toxicity was also tracked through mitochondrial changes and ATP assay (Mbye et al., 2008).

**Results:** Endothelial cells differentiated from iPSC cells expressed CD31 and VE-Cadherin as endothelial cells markers

and showed functional characteristics using Matrigel assay. CSA showed EC50 around 5µM on endothelial cells viability. Mitochondrial experiments proved a deficiency in mitochondrial complexes of endothelial cells after CSA treatment. Also, CSA treatment could increase ATP synthase in our cells which altogether led to impaired angiogenesis checked by sprouting assay.

**Discussion and Conclusion:** So, by now, we could design an *in vitro* model of endothelial cells help us finding out exact toxicity of CSA on endothelial cells focusing on angiogenesis impairment in patients (Nowak-Sliwinska et al., 2018).

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**Presentation:** Oral

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## Human immunocompetent choroid-on-chip: A promising tool for studying ocular side effects of biological drugs

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Next-generation therapies such as complex biologics are associated with ocular side effects in up to 70% of patients (Hager and Seitz, 2013). The immune-protective role of the choroid has been recognized as a key element for the maintenance of ocular

health (Chen et al., 2019; Jehs et al., 2016). Non-clinical investigations of the underlying molecular mechanisms and immune responses are complicated by interspecies differences of the immune system (Bjornson-Hooper et al., 2019) and the target homology as well as class-related properties of biologics and immunomodulatory drugs. We have developed a human cell-based *in vitro* model of the choroid layer of the eye based on a tailored microfluidic platform: Our choroid-on-chip integrates key choroidal cell types – melanocytes, retinal pigmented epithelial cells and microvascular endothelial cells – in a microphysiological environment mimicking *in vivo* tissue architecture and vasculature-like perfusion (Probst et al., 2018). Cell identity, viability and function are maintained for up to 2 weeks in perfused culture. Immunocompetence is achieved by perfusion of immune cells (PBMCs) through the endothelial channel. To demonstrate controlled immune cell recruitment into the melanocyte compartment through the vascular monolayer, we exposed the system to the immunomodulatory drug cyclosporine in combination with anti-CD3/CD28 stimulation. By monitoring cytokine secretion and quantitative 3D image analysis, we could evaluate the immune response *in situ*. The stimulation led to cell activation and increase in IFN $\gamma$ , TNF $\alpha$ , Granzyme B and IL-2 levels, which was reversed in a concentration-responsive manner by the exposure to cyclosporine. Similarly, immune cell recruitment was induced by anti-CD3/CD28 activation and prevented by cyclosporine. This human immunocompetent Choroid-on-Chip offers a platform for profiling of selected drug candidates to inform early on choroid safety liabilities and guide the risk mitigation strategy prior to first-in-human studies.

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**Presentation:** Oral

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## Secretome characterization of 3D bronchial epithelial cultures to study the role of protein corona on the fate and long-term effects of nanoparticles

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Over the last years, the production and use of nanomaterials (NMs) in industry has increased exponentially becoming a potential hazard for human health. In order to reduce animal testing, alternative methods have to be developed to characterize NM hazard. The airway epithelium constitutes the first barrier in the lung against inhaled NM absorption and understanding the mechanism of entry of NMs in the airway epithelium could help us to elucidate the way to avoid NM toxicity.

The growth of epithelial cells on a Transwell® membrane allows the differentiation and the establishment of a 3D *in vitro* epithelial barrier (Grainger et al., 2006) which can be used to study NM internalization and long-term toxicity after repeated exposure. NM toxicity does not depend only on NM-cell interactions but also on their interaction with molecules in the biological fluid they encounter (Raesch et al., 2015). To characterize the protein corona and its role in NM interactions with respiratory cells, we have developed long term 3D cell culture composed of Calu-3 cells growing at the air-liquid interface on Transwell® inserts. The air liquid interface allows the production of proteins into the apical part resembling the biological fluid present in the lung. The composition of the apical secretome of Calu-3 cells has been compared with the secretome produced by primary normal bronchial human epithelial (NBHE) cells and human bronchial alveolar lavage (hBAL) presenting 97% and 30% common proteins respectively.

The Calu-3 apical secretome have been used to characterize the proteins absorbed on silver nanoparticles. The analysis of absorbed proteins by SDS-PAGE and mass spectrometry will bring new knowledge to understand the role of a specific protein corona formed in biological fluid in the fate and effects of NMs.

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**Presentation:** Oral



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## The ongoing journey to champion and enhance a culture of care

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Culture of care is a well-established regulatory requirement in the UK. The publication of the RSPCA and LASA guidance “Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies” in 2015 articulated guiding principles on how to promote a culture of care and the UK Home Office’s expectation for establishments breeding, supplying and using animals in research is to not only promote but to champion, nurture and enhance a culture of care. Over the last 5 years tools, advice and guidance on culture of care have become available however, establishing and defining what a culture of care means to you and your place of work is the starting point before continuing on the journey to champion, nurture and enhance the culture. This is and will be a unique journey for everyone, even though we may all apply the same or similar key principles in shaping our cultures. GlaxoSmithKline are currently on this journey both in the UK and globally. This session will share some of our key experiences, the good, the bad and the ugly in the hope you can derive some benefit to help you on your journey and that we, in turn, may learn from your experiences.

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**Presentation:** Oral

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## Promoting non-animal approaches within the EU chemicals strategy for sustainability

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The European policy framework for chemicals is recognized as the most advanced and evidence-based globally, although current and future challenges call for an even stronger capacity and leadership. In line with the European Green Deal, the Chemicals Strategy for Sustainability of the European Commission promotes a vision of a toxic-free environment, where chemicals are produced and used in a way that maximizes their contribution to

society while minimizing their harm to current and future generations. The Strategy sets out five overarching priorities for the EU chemicals policy:

1. Strengthen the protection of human health and the environment from chemical risks
  2. Catalyze the shift to safe and sustainable innovation and foster EU competitiveness
  3. A more coherent and effective legal framework on chemicals
  4. Provide a comprehensive and transparent knowledge base for citizens, authorities and industries
  5. Provide a model inspiring chemicals management globally
- Within these priorities, especially priority 3, the strategy also aims to reduce dependency on animal testing by promoting multidisciplinary research and ensure the continued development and regulatory uptake of new and innovative tools – including Virtual Human Platforms – to improve quality, efficiency and speed of hazard and risk assessment on humans, ecosystems and specific population groups. In addition, within priority 5, the strategy proposes to promote common standards and innovative risk assessment tools internationally (e.g., in OECD) to shift away from unnecessary animal testing (e.g., adapt the UN GHS criteria to alternative methods).

The presentation will briefly explain the EU Chemicals Strategy for Sustainability and highlight how the use of non-animal alternative approaches can be increased at the same time.

**Presentation:** Oral

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## Regulatory science: Industry, research and innovation for the testing of substances

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Regulatory science covers the performance of studies and tests for regulated substances, and their interpretation by experts and scientists of regulatory agencies, to decide on the standards that should apply to the substances in question. Regulatory science is frequently depicted as a separate realm of science, disconnected from fundamental research. It is criticized for being a conservative area of scientific work, in which well-established protocols are routinely applied, with only slow integration of emerging new techniques and results from research. For some, this is evidence of the fact that regulatory science is too heavily controlled by the regulated industries, and an inadequate tool to reveal risks and support protective policies. On the other hand, regulatory science is now emerging as an area of interest for top universities. New research centers are being set up, benefiting from new sources of funding. Alternative methods



make their way, progressively, into recommended test batteries. This session will be devoted to exploring and discussing the role that research and innovation plays, or ought to play, in regulatory science.

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**Presentation:** Oral

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### Combining mathematics with medicine to make better use of animal data: Sepsis case study

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Sepsis is a major worldwide healthcare issue with unmet clinical need. Despite extensive animal research in this area, successful clinical translation has been largely unsuccessful. As sepsis models can lead to a rapid and substantial suffering – it is essential that we continually review experimental approaches and undertake a full harm:benefit impact assessment for each study. In some instances, this may require refinement of existing sepsis models. In other cases, it may be replacement to a different experimental system altogether, answering a mechanistic question whilst aligning with the principles of reduction, refinement and replacement (3Rs). Finally, it is essential we make better use of clinical data to inform our research. Cardiovascular decompensation is a hallmark feature of clinical sepsis. Cardiovascular parameters are routinely monitored at high fidelity in conscious animals, but much of the data is underused. Through an interdisciplinary collaboration, we have combined expertise from biomedicine and mathematics to develop a new mathematical technique (Symmetric Projection Attractor Reconstruction – SPAR) that can extract more information from physiological waveforms such as blood pressure. I will demonstrate how such approaches can both extract more information from collected signals and also increase the sensitivity of detecting changes. This in turn may lead to refinements (e.g., earlier, more humane end points) or allow scientists to make better use of existing data, reducing the need for new procedures. Finally, I will show how we have used our mathematical findings in retrospective research animal data to support our analysis of retrospective clinical data. In short, we demonstrate that SPAR has the potential to extract more information about cardiovascular health which in turn could support clinical decision making.

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**Presentation:** Oral

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### From microphysiological to micropathophysiological systems to study neurotoxicity and CNS diseases

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Exposure to environmental chemicals during early life is suspected to contribute to the increasing incidence of neurodevelopmental disorders, especially autism. Currently, 1 in 59 children in US are diagnosed with autism spectrum disorder (ASD), and this cannot be explained only by the genetics, suggesting that environmental exposures contribute. Demanding animal tests for Developmental Neurotoxicity (DNT) have been devised, but because of complex underlying mechanisms, limitations of current approaches are enormous. The high costs and technical difficulties of these tests are prohibitive for routine DNT chemicals screening. Alternatives are needed to change this. We developed in our laboratory human iPSC-derived brain model (BrainSpheres) to study not only effects of chemical exposure on brain developmental and neurogenesis, but also neural disorders and gene environmental interactions leading to such disorders as autism, ALS, MS, Parkinson's and Alzheimer's.

**Presentation:** Oral



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## **In silico approaches to link adverse outcomes to molecular initiating events through AOPs**

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The Adverse Outcome Pathway (AOP) provides a framework to encapsulate the chemical and biological processes that can lead to toxicological outcomes. The Molecular Initiating Event (MIE) is the initial chemical interaction that starts in an AOP. Understand the chemistry behind MIEs is key to predicting molecular toxicity *in silico*.

To build computational models for MIE prediction we use a variety of algorithms. 2D structure-activity relationships are a transparent and clear link between molecular structure and biological activity. Machine learning algorithms such as random forests and neural networks are excellent predictors but tend to lack this transparency. Using these methods in combination is a good way of making the best of all their strengths. Combined models for human MIE prediction have been constructed using open-source data from ChEMBL and ToxCast and are being integrated into safety decision-making procedures at Unilever. These predictions can be linked to adverse outcomes using AOPs published in the AOP Wiki.

To further explore the interactions behind MIEs we have used quantum mechanical and comparative molecular field analysis (CoMFA) calculations. These have allowed us to directly observe the interaction between DNA and an electrophilic chemical and explore how chemicals bind to receptor pockets. Activation energies were found to correlate well with Ames mutagenicity experimental values, and CoMFA provides estimations of molecular activity at targets and fields that can be visualised to see how interactions happen. These calculations allow us to better understand how MIEs happen, and how molecules can be modified to avoid them.

AOP based risk assessment requires a large amount of knowledge across a wide area of biological space. Predictive computational methodology can help, particularly at the level of the MIE where the interaction can be well modelled, and will benefit from additional AOP development, linking predicted MIEs to adverse outcomes.

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**Presentation:** Oral

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## **Computational intelligence: Opening DART's "black-box" with agent-based models**

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New Approach Methodologies (NAMs) for toxicity assessment that are based largely on *in vitro* data and *in silico* models provide a path forward to animal-free testing of the potential for developmental and reproductive toxicity (predictive DART). A major science challenge is translating complex data and information into predictive models for human toxicity. For predictive DART, this means virtually extrapolating data from *in vitro* studies with human stem cells and related systems into adverse developmental outcome(s) of relevance to regulatory decision-making. Computational agent-based models (ABM) of morphogenetic systems have distinct advantages for this purpose. ABMs offer unparalleled flexibility for multiscale modeling of tissue dynamics. Nature-inspired agents (cells) and rules (behaviors) are set into motion with soft-computing. Fuzzy logic is utilized to simulate forces or properties governing cell fate and behavior where rules are inexact or knowledge incomplete. ABMs can change course in response to a specific situation or stimulus from genetic and/or environmental cues from real world data such as *in vitro* high-throughput screening (HTS) yielding a probabilistic rendering of where, when and how a particular condition might lead to an adverse developmental outcome (cybermorphs). Opening the black-box of DART with computational intelligence comes with key challenges for science and technology development. Does not reflect Agency policy.

**Presentation:** Oral



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## An integrated approach to testing and assessment for evaluating inhalation risk

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The utility of conventional *in vivo* animal studies to characterize toxicity associated with repeat inhalation exposures to irritants is limited by the complexity of extrapolation to humans, as well as by animal welfare concerns. Recent developments in *in vitro* technologies have produced systems which provide functionally accurate representations of the human respiratory tract and can offer more relevant measures of human toxicity. In this study, an *in vitro* model of the human upper airway epithelium (MucilAir™) has been evaluated for a fungicide (chlorothalonil) which exhibits irritant properties in mucous membranes *in vivo* through localized cytotoxicity. As these responses occur locally at the respiratory portal of entry, they are particularly amenable to modelling *in vitro*. A number of endpoints were assessed with MucilAir™ (trans-epithelial electrical resistance, lactate dehydrogenase release and resazurin metabolism) as markers of local irritation. The MucilAir™ results showed a good concordance of response across endpoints and across human tissue donors. A wide range of concentrations were tested in order to derive points of departure for risk assessment via benchmark dose modelling. In addition, anatomically and physiologically correct 3D, computational fluid-particle dynamics (CFPD) models were developed to estimate airway surface deposition of inhaled aerosol formulations of chlorothalonil in discrete regions of the upper conducting airways. This work demonstrated that the combined use of three-dimensional *in vitro* and *in silico* models of the human respiratory epithelium and tract are a viable alternative for quantitative assessment of short- and long-term inhalation exposure to irritant materials.

**Presentation:** Oral

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## A 21<sup>st</sup>-century roadmap for biomedical research and drug discovery: Recommendations

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Current animal-based research methods can take up to ten years and billions of dollars (DiMasi et al., 2016) to develop a single pharmaceutical which even then may have a 90% failure rate when it reaches human trials. It is a highly inefficient model that has seen

the number of new drugs reaching the market plummet in recent years and it appears that if we continue on this path, we will have no more new drugs after the year 2070 (Scannell et al., 2012).

A paradigm shift is on the horizon, however. Advances in biotechnology, microphysiological systems, systems biology, pathway-based approaches, and computer modelling are leading a revolution in drug safety testing. In addition to these innovations, there is a growing recognition among scientists that to improve the success rate of drug candidates, a stronger focus is needed on human-relevant data (Marshall et al., 2018).

Here, we will discuss existing efforts to prioritize human-based biology for health research and propose key recommendations to revitalize the drug discovery process. We consider ongoing efforts to collect human-relevant data and what will be required to successfully apply these to enhance drug development productivity. We look at the need for greater interdisciplinary and international collaboration, incentivization of global data sharing, and for global funding calls to prioritize human-based methods. Innovative models such as induced pluripotent stem cells, organoids and organs-on-chips are the future of successful and personalized drug development, but only if we support and nurture these approaches.

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**Presentation:** Oral

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## An evaluation framework for new approach methodologies (NAMs) for human health safety assessment

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The need to develop new tools and increase capacity to test pharmaceuticals and other chemicals for potential adverse impacts on human health and the environment is an active area of development. Much of this activity was sparked by two reports from the US National Research Council (NRC) of the National Academies of Sciences, Toxicity Testing in the Twenty-first Century:



A Vision and a Strategy (2007) and Science and Decisions: Advancing Risk Assessment (2009), both of which advocated for “science-informed decision-making” in the field of human health risk assessment. The response to these challenges for a “paradigm shift” toward using new approach methodologies (NAMS) for safety assessment has resulted in an explosion of initiatives by numerous organizations, but, for the most part, these have been carried out independently and are not coordinated in any meaningful way. To help remedy this situation, a framework that presents a consistent set of criteria, universal across initiatives, to evaluate a NAM’s fit-for-purpose was developed by a multi-stakeholder group of industry, academic, and regulatory experts. The goal of this framework is to support greater consistency across existing and future initiatives by providing a structure to collect relevant information to build confidence that will accelerate, facilitate and encourage development of new NAMS that can ultimately be used within the appropriate regulatory contexts. In addition, this framework provides a systematic approach to evaluate the currently available NAMS and determine their suitability for potential regulatory application. This 3-step evaluation framework along with the demonstrated application with case studies, will help build confidence in the scientific understanding of these methods and their value for chemical assessment and regulatory decision-making.

**Presentation:** Oral

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## Setting the scene: New approach methodologies (NAM)-supported read-across approaches

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Read-across is one of the most frequently used alternative tools for hazard assessment, in particular for complex endpoints such as repeated dose or developmental and reproductive toxicity. Read-across extrapolates the outcome of a specific toxicological *in vivo* endpoint from tested (source) compounds to “similar” (target) compound(s). If appropriately applied, a read-across approach can be used instead of *de novo* animal testing. The read-across approach starts with structural/physicochemical similarity between target and source compounds, assuming that similar structural characteristics lead to similar human hazards. In addition, similarity also has to be shown for the toxicokinetic and toxicodynamic properties of the grouped compounds.

EU-ToxRisk has developed a general outline for a read-across assessment concept using New Approach Methodologies (NAMS), to support hazard characterization of the grouped compounds by generating data on their dynamic and kinetic properties. This NAM-based strategy has been published as a white

paper in Archives of Toxicology (Escher et al., 2019). Based on the overarching read-across hypothesis, the read-across workflow suggests targeted or untargeted NAM testing also outlining how mechanistic knowledge such as adverse outcome pathways (AOPs) can be utilized. Toxicokinetic models, enriched by *in vitro* parameters such as plasma protein binding and hepatocellular clearance, are proposed to show (dis)similarity of target and source compound toxicokinetics. Furthermore, *in vitro* to *in vivo* extrapolation is proposed to predict a human equivalent dose, as potential point of departure for risk assessment. Finally, the generated NAM data are anchored to the existing *in vivo* data of source compounds to predict the hazard of the target compound in a qualitative and/or quantitative manner.

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**Presentation:** Oral

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## A human xenobiotic metabolic system adapted to quantitative high-throughput screening processes

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The evaluation of the toxicity of chemical compounds remains, to a large extent, based on animal experimentation, using high doses associated with the observation of deleterious endpoint effects, and then extrapolating these effects to the human situation at low doses. These approaches are not only very expensive in time and cost but use a large number of animals to give toxicity results that are not robust and are not readily extrapolatable to humans. As well, these low throughput approaches are not adapted to the evaluation of the thousands of chemicals to which the population is exposed. Currently, toxicology is therefore faced with a two-fold challenge: to develop *in vitro* models that are as predictive as possible for human toxicity, and also that these tests be adaptable to high throughput screening approaches. Indeed, the majority of tests currently used for the evaluation of toxicity are lacking in metabolic capacity, while metabolism, in particular hepatic metabolism, plays a central role in the toxic response in the detoxification, as well as the bioactivation of xenobiotic compounds. There is therefore an urgent need to develop models which integrate a metabolic component in the context of high throughput *in vitro*



testing. We studied the integration of an exogenous human hepatic metabolic system adapted to high throughput *in vitro* testing by using either 2D or 3D HepaRG in high throughput toxicity tests. We used conditioned medium transfer strategy, test compound metabolism occurs separately and prior to assays and media containing metabolites from HepaRG cells were transferred to target cells for qHTS assays (cytotoxicity, genotoxicity and nuclear receptors activation).

**Presentation:** Oral

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## Of mice and not men: Lessons from academia

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Animal models continue to serve as the primary, and sometimes only, mode of investigation to elucidate human biological processes in health and disease. The conserved nature of biology has often been interpreted or taken for granted that humans and experimental animals are identical at the molecular, cellular, organ, and systems levels. But is it legitimate to assume that observations in experimental animals can be extrapolated to the situation in humans? I will discuss this problem taking the pancreatic islets (that secrete the blood glucose-regulating hormone insulin) an example. Until recently, almost all studies on the regulation of insulin secretion were conducted using isolated islets from mice or rats. As a by-product of the successful introduction of clinical islet transplantation programmes ~20 years ago, isolated human islets sometimes become available for research (quality/quantity insufficient for transplantation). This has given us an opportunity to test the hypothesis that rodent islets are useful models of human islets. Work in several laboratories has now illustrated important structural, functional and genetic differences between rodent and human islets (Rorsman and Ashcroft, 2018). The inconvenient conclusion emerging from these studies is that “human islets are the best model of human islets”; rodent islets should be viewed as Ersatz of the real thing. This does not mean that rodent islets are useless as a model, but studies must take the origin in observations made in whole-body human physiology and isolated human islets: once an interesting process has been identified, the finer details can be explored in (humanised) rodent islets. Access to human islets remains limited and many experiments are not feasible in human islets. However, with the realization of the numerous and important differences between rodent and human islets, it is no longer permissible to make bold claims about human pathophysiology based solely on observations in rodents.

## Reference

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**Presentation:** Oral

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## US EPA adverse outcome pathway database (AOP-DB) semantic integration and workshop opening remarks

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There is a need for more efficient use of existing data to characterize human toxicological response data for environmental chemicals of interest to the US Environmental Protection Agency (EPA). The Adverse Outcome Pathway (AOP) framework helps to organize existing mechanistic information, where AOP data is currently submitted directly by users and stored in the AOPWiki. Automatic and systematic parsing of AOPWiki data is challenging, so we have created the EPA AOP-DB. The AOP-DB is an AOP profiler, developed to assist in biological and mechanistic characterization of AOP data and provide a broad, systems-level overview of the biological context of AOPs. We describe here recent semantic mapping efforts for the AOP-DB, as part of the EU funded OpenRiskNet collaboration, and how this process integrates AOP-DB data with other toxicologically relevant datasets. Finally, we open session with the discussion on how semantic integration of toxicologically relevant, biological data can further the mission of the EPA to protect human health and the environment.

**Presentation:** Oral

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## NIH replacement for human rabies vaccine: Method development and strategy for implementation of new ELISA for commercial product

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Sanofi Pasteur has developed and validated an ELISA to detect and quantify rabies virus Glycoprotein G structural alterations (Chabaud-Riou et al., 2017). This ELISA is based on two neutralizing monoclonal antibodies targeting the well-conformed



G-protein sites II (Gamoh et al., 1996) and III (Nagarajan et al., 2006). Both sites have been identified as important in triggering a protective immune response against rabies. To demonstrate that this ELISA may be a good candidate for the replacement for the NIH potency test (lethal challenge assay in mice), several studies have been conducted that demonstrate the ability of the ELISA to monitor the potency of the vaccine. Thermo-degraded and hyper-inactivated rabies were analyzed with both the ELISA and NIH tests (Toinon et al., 2019), the results showed an agreement between the ELISA and NIH results however, the ELISA is more precise in detecting structural alteration of the virus than the NIH test. Considering this data, this ELISA has been selected by EDQM (European Directorate for the Quality of Medicines) for a collaborative international study (Morgeaux et al., 2017) (Biological Standardization Program 0148 study) to replace the NIH test.

To implement this ELISA on routine basis, different strategies were set up to define new acceptance criteria for commercialized Verorab<sup>®</sup> vaccine.

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**Presentation:** Oral

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## Towards biological plausibility using linked open data

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Behind risk assessment is experimental evidence. Behind biological knowledge is primary literature. However, because the amount of knowledge keeps growing, our experimental technologies are advancing and getting increasingly complex, even experts no longer can keep up with the progress in mechanistic understanding, outside their increasingly specialist domain. At the same time, the number of biological questions with a simple answer keeps dropping and many modern questions have complex answers. Access to the right facts at the right time needs a change of thinking.

The idea of linking facts and data at a large scale was envisioned long ago, but only recently became viable, with the in-

roduction of the semantic web and linked open data. These new technologies make it possible to easily link remote knowledge, taking advantage of globally unique identifiers and exact meaning with ontologies (Samwald et al., 2011; Willighagen et al., 2013). This presentation outlines how we applied these ideas to the life sciences in general and with applications to toxicology. Using eNanoMapper (Hastings et al., 2015), WikiPathways (Waagmeester et al., 2016), and Wikidata (Waagmeester et al., 2020), it will show how semantic web approaches can be used to answer questions that are much harder to answer with older approaches. Examples will show 1. how we can use SPARQL to return all assay experiments for all types of metal oxides, 2. how biological pathway knowledge can be combined with knowledge from chemical databases, and 3. how we can find research about and scholars that study particular genes, proteins, or toxicants.

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**Presentation:** Oral

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## Cosmetics Europe validation studies of *in vitro* skin genotoxic assays and their use in a strategy to support the safety assessment of cosmetic ingredients

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The EU Cosmetics Directive prohibits the use of *in vivo* genotoxicity models and, while the *in vitro* 2-test battery has a high sensitivity for prediction of *in vivo* genotoxic/carcinogenic agents, it tends to over-predict the genotoxicity hazard, resulting in mis-



leading positive results. To address this, the Cosmetics Europe Genotoxicity Task Force has established two *in vitro* skin genotoxicity models as follow up assays to the 2-test battery for substances with dermal exposure: the reconstructed skin (RS) Comet assay and the RS micronucleus (RSMN) test. Here, we report on the completed validation of these assays. Both assays exhibited good sensitivity and specificity: 77% and 88% for 3D Skin Comet (32 compounds) and 75% and 84% for the RSMN (43 compounds) (Pfuhrer et al., 2021a,b). A combination of these assays enables detection of DNA damage leading to all 3 types of genotoxic damage (mutation, clastogenicity and aneugenicity). In the validation dataset, most of the true positive chemicals were positive in both assays of the 2-test battery, therefore, the results of both the RSMN and the 3D Skin Comet assay need to be considered to make a final call on the chemical's genotoxic potential. By applying this endpoint-triggered strategy, the sensitivity increased to 89%.

In conclusion, the high predictivity of the expected genotoxicity *in vivo* observed for these higher tier *in vitro* assays supports their use as follow-up tests to the standard 2-test battery. For topically applied chemicals, the RSMN assay is recommended for *in vitro* micronucleus positive chemicals, whereas Ames positives should be followed-up with a RS Comet assay. This tiered strategy shows great promise as an *in vitro*-only approach for genotoxicity testing of cosmetic ingredients. Both assays were accepted into the OECD guideline development program.

*Funding: Cosmetics Europe and the German Ministry for Research and Education.*

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**Presentation:** Oral

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## Quantitative prediction of developmental toxicity by modelling the DARTable genome

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Adverse outcome pathways (AOP) describe the series of necessary, measurable key events starting at a molecular initiating event (MIE) that result in an adverse outcome, i.e., it describes the realization of a pathological process resulting in a pathological phenotype. We need to know 3 things to predict the dose required for a chemical treatment to cause an adverse event: the set of MIEs that could produce a development or reproductive toxicity (DART), called the "DARTable genome"; the disposition of a chemical to participate in a pathological process by interacting with and modifying the activity of the DARTable genome; and whether the concentration of the chemical at the site of the MIE exceeds some critical threshold necessary to trigger the pathological process. Data structures and ontologies that contain these concepts will enable the integration of the known toxicology, pharmaco-/toxicokinetic and protein-interaction information. From this we can identify the set of known MIEs and simultaneously derive the threshold. The hypothetical DARTable genome can be extended by including genes with developmental defects when knocked out in model species or by using machine learn to identify properties of DARTable genes. Additionally, when the MIEs are unknown, but other key events are, the relationship between the key event and the threshold can be determined. QSAR models can predict whether a chemical participates in a MIE. However, chemical-protein interaction data are sparse for most targets so transfer learning will be needed to generalize this to the entire DARTable genome. Omics-based methods may help determine thresholds for targets with unknown MIEs. But suitable analysis methods and annotated repositories are not yet available. Most challenging is the determination the threshold without *in vivo* studies? Advances in modelling biological processes at an appropriate level of detail are required to fully realize this approach.

**Presentation:** Oral



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## A new *in vitro* model for interrogating DILI susceptibility for patients with benign fatty liver disease

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Obesity and related metabolic disorders, such as type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), coronary heart disease and cancer are a major and growing health burden in modern society. Obese patients consume on average more drugs than non-obese individuals and are at higher risk to suffer from drug-induced liver injury (DILI), not only due to increased adverse drug-drug interactions, but also due to enhanced susceptibility related to metabolic disturbances frequently observed in obese patients. To address toxicity liabilities in patients with metabolic liver disorders, we aimed to develop an *in vitro* model based on 3D liver spheroids by recapitulating disease specific features similar to the clinical hallmarks of NAFLD. Characterization and metabolic performance data will be presented on a *in vitro* model representing benign fatty liver, the most relevant pathophysiological model representing a sizeable fraction of the population under drug administration. The model will serve as a toolbox to simulate the different stages of NAFLD, including NASH and fibrosis, and to test associated risks upon exposure to compounds under investigation.

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**Presentation:** Oral

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## A high-throughput, microfluidic platform for drug screening on vascularized 3D tissues

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3D tissues such as spheroids or organoids derived from human stem cells represent a new type of three-dimensional *in vi-*

*tro* model for understanding organ development, disease mechanisms, and drug testing. Despite the success in generating 3D cultures resembling different tissue types (brain, heart, intestine, liver, lung, and kidney), these mini-organs show limited growth potential and an immature phenotype, with a lack of vascularization being one of the leading causes. We recently described the use of the Organoplate<sup>®</sup> for generating 3D perfused, angiogenic vessels. Here, we present the use of the Organoplate<sup>®</sup> Graft, a high throughput “grafting” platform, which allows blood vessel co-culture with 3D tissue aggregates and tissue vascularization. We propose the use of the Organoplate<sup>®</sup> Graft as a grafting platform for multiple 3D tissues allowing drug screening and disease modeling in a more physiologically relevant environment.

### Reference

Kurek et al. (in prep). High throughput microfluidic platform for 3D tissue vascularization.

**Presentation:** Oral

869

## CRACK IT: 3D hiPSC-derived laminated retinal model as a tool for toxicology and drug discovery studies

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Retinal toxicity, when observed preclinically, can be a serious hurdle for drug development. Assessment of retinal alteration and mechanistic investigations *in vivo*, are challenging. In addition, the translation from preclinical animal species to human safety is equally delicate and lacks adequate tools. Hence, there is an urgent and unmet need for *in vitro* models that can recapitulate human retinal physiology and function. Here, we describe a consortium working through the NC3Rs CRACK IT Challenge programme (<https://nc3rs.org.uk/crackit/>) that is developing a physiologically competent human retinal cell model. Retinal organoids (RO) derived from induced pluripotent stem cells (iPSCs) provide a physiologically relevant human platform to study retinal development, disease modelling and compound screening. We have developed a protocol for efficient generation of ROs from human iPSCs in 96-well plates to allow large-scale production. ROs contain all the major retinal cell types and when



subjected to known retinotoxins produce a response in a predictive manner. The team are investigating methods for transporting ROs by verifying their tolerability to different temperatures. The Challenge team comprises of the NC3Rs, who fund and facilitate the programme, industry sponsors who set the Challenge – Merck, Novartis and Roche – and a team of contractors, Newcells Biotech Ltd in collaboration with Newcastle University, who deliver the work. Sponsors provide expertise in industry specifications for a model that is fit for purpose and in-house testing of the models to provide cross centre early-stage validation to aid in getting the product to market. The team will present the benefits of the CRACK IT Challenges approach and the scientific and technical advances delivered that will provide significant animal replacement opportunities for the study of retinal toxicity and ophthalmologic disease.

**Presentation:** Oral

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## Alternatives for animals in drug discovery research: Religion interface and current trends in culture of care and alternatives to experiments on animals

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We want to enable research and disrupt the current early drug discovery programs with new technologies. This facilitates replacing animals in research and cuts down the costs immensely, apart from finding many aspects of drug metabolites, safety, toxicity, transport mechanisms and DD interactions. All these, done with one thing in our minds, and that is to consider the moral and ethical reasons on why and how we should use these animals in research.

Many religions promote good and compassionate animal care, it be domestic or wild. The current world uses lab animals to discover new drugs and test toxicities of existing ones. A healthy research program exists if all these religions continue to promote such compassion towards animals. Culture of care is one such educated care that can promote the care of animals.

In the context of this conference, any step towards finding and using an alternative is a reflection of how we think about animals in research. Scientists are in the process of adding new platforms towards eliminating critical hurdles and enhancing translation across the R&D value chain. These platforms would eventually benefit pharma, agrochemicals, cosmetics, and nutraceuticals. Assays on such platforms would inspire dynamic and real world use them in a highly reliable manner. In essence, such platforms

would de-risk clinical and product failures early on by improving the translatability of many assays and can easily become a genuine, cost-effective and more predictive alternative to testing in animals.

As many scientists and companies work on these disruptive technologies to replace animals in research, it is imperative that lab animal scientists focus on continuous refinement and fine-tuning of experiments with animals.

**Presentation:** Oral

876

## Organoids: Less animal studies, more relevant data

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Organoids such as adult epithelial stem cell derived organoids are proving to be a major breakthrough in preclinical models. The new patient-like models are fundamental change in the way drug discovery and development can be performed. The development of the organoids started with the discovery of the identity of adult stem cells in human epithelial tissues such as intestine and liver. With the identification of these stem cells, we were able to develop a culture system that allowed for the virtually unlimited, genetically and phenotypically stable expansion of the epithelial cells from animals including humans, both from healthy and diseased tissue.

Recently, we and others have demonstrated that the *in vitro* response of organoids correlates with the clinical outcome of the patient from which the organoid was derived. In addition, we have developed a coculture system using HUB Organoids and the immune system to study this interaction and drugs that target the role of the immune system in cancer and other diseases. Novel models and assay can be used to study intestinal and lung barrier function and transport of the epithelium of these organs. These experiments show how HUB Organoids can be used to study mechanism that underly barrier function disruption in IBD or COPD. Furthermore, we have developed new models to study the interaction between immune system and epithelium. The combination of the new coculture models and assay development to study the epithelium allows us new insights into disease mechanisms and drug treatment strategies.

**Presentation:** Oral



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## Multi-scale, virtual-tissue simulations of developmental toxicity and toxicology as an approach to minimize the required number of animal experiments

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Our ability to integrate molecular and genetic information to make biomedically meaningful predictions of developmental toxicity and toxicology in adults at the organ or organism level is still limited, because of the difficulty of predicting the emergent properties of tissues from cells' molecular signatures. Extrapolating experimental findings from animals to humans or from organoid culture or other *in vitro* assays to humans is also problematic. Virtual-Tissue toxicology simulations are mechanistic computer simulations which attempt to represent the complex molecular processes inside cells, the structural complexity of tissues and their responses and the organismic context of those tissues. Validated Virtual-Tissue models maximize the amount of knowledge derived from individual animal experiments, allowing their number to be reduced and improve our ability to make human-relevant predictions from toxicology and drug-discovery experiments in fish and *in vitro* assays. For the past 20 years, we have been developing computational tools and approaches to bridge the gap between molecule and physiological outcome and to make these computational methodologies more accessible to experimentalists, clinicians and regulators. Our open-source CompuCell3D modeling environment enables rapid specification and refinement of complex biomedical computer simulations that combine subcellular molecular reaction-kinetics models, the physical and mechanical behaviors of cells and the longer-range effects of the extracellular environment. I will specifically focus on the types of questions that simulations can address and the types of experimental data required for their development and validation and illustrate these approaches using our computational studies of the mechanisms of acetaminophen toxicity in the liver (Sluka et al., 2016; Fu et al., 2018) and of Polycystic kidney disease (Belmonte et al., 2016, where we used these simulations in the context of drug discovery).

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**Presentation:** Oral

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## Advanced human 3-dimensional test systems paired with modern exposure systems: Progress toward recreating physiological-like inhalation exposures

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With the increase in chemicals and materials that may cause pulmonary toxicity through inhalation, a need for cost-effective toxicity testing of new chemicals continues to grow. The 2007 report, "Toxicity Testing in the 21<sup>st</sup> Century – a Vision and a Strategy", The National Academies Press, Washington D.C., describes a path forward for toxicology and envisions the use of more predictive human-relevant *in vitro* models for estimating human health risks. The ethical considerations, poor predictive value of animal testing, and vast numbers of chemicals/materials requiring evaluation further drives this argument for the application of *in vitro* models to assess pulmonary toxicity.

*In vitro* models utilizing human-derived cell lines and primary cells provide endpoints that reflect cytotoxic, genotoxic, and other relevant adverse events following exposure to toxicants. The emergence of multicellular three-dimensional (3D) tissue culture systems of the respiratory tract provides toxicologists with test platforms that accurately model the complex processes observed in native tissues. Human donor-tissue derived spheroids/organoids, reconstructed human airways, and precision-cut lung slices provide conventional toxicity endpoints as well as complex, relevant events following chemical exposure. The varied cell types, physiological structure, relevant toxicokinetics, and other properties of these models allow additional evaluations ranging from chronic-exposure related events (e.g., persistent inflammation, goblet cell hyperplasia) to functional outcomes (e.g., ciliary beating assays) that can reflect serious health complications that may lead to chronic obstructive pulmonary disease.

This presentation provides an overview of *in vitro/ex vivo* pulmonary models and their incorporation into a pragmatic screening/testing strategy for materials that may induce complex human pulmonary adverse events.

**Presentation:** Oral



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## Beyond program review/post approval monitoring: Developing and implementing a quality improvement program for laboratory animal research

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A high functioning IACUC not only maintains its operations with integrity and efficiency, it also undergoes continuous quality improvement. More than the required program review or post approval monitoring, this presentation will offer a discussion of how an IACUC office has developed and implemented programs of quality improvement in its animal research program including both facility and IACUC offices – QI to improved compliance and/or efficiency and effectiveness. Specific topics include selection of areas to focus on quality improvement (QI) efforts, implementation of QI activities and programmatic outcomes of QI efforts. Fundamentally, the presentation will describe and assess experiences with QI activities.

**Presentation:** Oral

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## Using a dry powder Vitrocell system to expose respiratory epithelial models

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In order to improve the relevance of respiratory hazard assessment of nanomaterials we have used a dry powder aerosol exposure system. Human airway cells (Calu-3) were exposed to an aerosol of dry powder ZnO (80 nm), CeO<sub>2</sub> (40 nm), TiO<sub>2</sub> (25 nm) or DQ12 (5 µm) and responses were compared to submerged culture exposures. Calu-3 cells were cultured on 3.0 µm pore size inserts until stable TEER values were obtained (7 days, typical values 700-1000 ohms per 1.12 cm<sup>2</sup>). The culture method was established as an SOP for the project <https://www.patrols-h2020.eu>

Cells were incubated for a further 7 days without culture medium on the apical side to create an air liquid interface (ALI). Cells received one or two 25-minute exposures of each particle type,

before incubating for a further 24 hours, still under ALI conditions. The supernatant in the basolateral chamber was sampled to assess cytotoxicity and IL-8 release.

Results indicate that both ZnO (140-458 ng/cm<sup>2</sup>) nanomaterials and DQ12 (respirable alpha-quartz) (80-662 ng/cm<sup>2</sup>) resulted in decreased cell viability compared with ambient air exposure. At the concentrations tested here was no difference in the impact regardless of whether the cells received one or two exposures. TiO<sub>2</sub> (38-185 ng/cm<sup>2</sup>) and CeO<sub>2</sub> (140-571 ng/cm<sup>2</sup>) nanomaterials did not decrease viability following either one or two exposures. IL-8 release was decreased by ZnO, increased by TiO<sub>2</sub> and not changed on treatment with DQ12 or CeO<sub>2</sub>. Our results suggest exposure using the dry powder system are in broad agreement with results obtained using submerged culture conditions. The dry powder system combined with the ALI Calu-3 culture is now suitable to test more concentrations and post-exposure times as well as for exposure of 3D multi-lineage respiratory models, providing the advantage of a more realistic exposure and assessment of dose to the cells.

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**Presentation:** Oral

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## A case study for the risk assessment of systemic toxicity

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Perhaps one of the most challenging areas for performing a risk assessment on a cosmetic ingredient in the absence of animal data is in fully assessing the toxicity risk from a cosmetic ingredient following systemic exposure. A broad range of *in vitro* and clinical studies are available to evaluate and provide information for use in risk assessment on skin and eye irritation, mutagenicity and genotoxicity and skin sensitization. However, and despite recent advances in the development of studies to support AOPs, there is still work to be done to fully and confidently address toxicity following systemic exposure.

The presentation will describe a tiered approach, building on exposure considerations, use of structure-activity information and read-across to build confidence in the safety assessment. The possibility to do additional studies, such as skin penetration, metabolism, use of AOPs and other *in vitro* assays is considered as well as some of the problems that may be encountered in the assessment. The limitations of this approach, in particular for more novel substances, is recognized. This will be illustrated through the use of a case study.

**Presentation:** Oral



887

## Application of new approach method for determining developmental toxicity

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The elimination of chemicals with developmental or reproductive toxicity (DART) is a critical design goal for crop protection active ingredients (AI). However, DART effects are difficult to predict given the diversity of biological mechanisms operating during ontogenesis. The ideal new approach would predict teratogenicity in humans and be fast, simple, reproducible, require small amounts of chemical and enable chemical design. To evaluate existing new approach methods, we conducted an exploratory programme for DART prediction. Wu et al. (2013) developed a decision tree to predict DART. Unfortunately, when applied to AIs the predictions are overly conservative. Including specific chemometric models using protein-chemical interactions and pharmacokinetic data improve the predictivity. *In vivo* tests using model organisms such as *C. elegans* or Zebrafish have also been proposed. For AIs we show these tests can identify some but not all chemical-class DART liability. However, they are unsuitable for replacement of DART studies due to poor concordance of predictions for individual compound results compared to rat and rabbit studies. Recently predictions using the DevTox quick predict test (Stemina) has been shown good performance. However, when considered by itself this test performs poorly for AIs. Again, performance is improved by correcting for steady-state systemic exposure. When an AOP is known, a combination of specific *in vitro* assays for the MIE and *in vivo* pharmacokinetic data predicts target-induced DART effects. However, generalising this approach requires we have quantitative understand of more DARTable genome MIEs. Once understood, *in silico* and high content *in vitro* tests could in theory identify these interactions. High throughput gene expression and BioMAPP Diversity Plus panel shows can give suitable mechanistic hypotheses. However, for most of the DARTable genome, reference chemicals are not present in the public L1000 datasets, reducing the utility of this approach until these gaps are filled.

**Presentation:** Oral

890

## Rigor, relevance and reproducibility in the use of *in vivo* models in the pharmaceutical industry

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*In vivo* models, especially so in the early stages of drug development are often criticized for their lack of translatability. There are several therapeutic areas where *in vivo* models have been and continue to be pivotal either directly as a translatable model or to demonstrate mechanism of action of various therapeutics. Furthermore, for immunogenicity, pharmacology, Biomarker evaluation, DMPK and preclinical toxicity studies, *in vivo* models continue to be an important part of drug development and often a regulatory requirement. It is now universally accepted that we should always actively explore better translatable, human relevant *in silico*, *in vitro* models and when *in vivo* models are warranted, they be conducted with the highest rigor, relevance and reproducibility. In focusing on the rigor, relevance and reproducibility, one shall be engaging statistical teams, leveraging multidisciplinary teams of medical/veterinary pathologists, bioimaging experts, transgenic modeling teams as well as scientific teams in a project centric manner. This integrated approach, along with clinical end point input from physicians (functional expertise), helps us to integrate the models into the process better. We attempt to constantly maximize the use of human relevant models (humanized models both *in vitro* and *in vivo*) in order to maximize the adherence to the 3Rs of animal research and when *in vivo* studies are absolutely warranted it is done with careful integrated approach keeping the Rigor, relevance and reproducibility as a key factor.

**Presentation:** Oral

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## Discussion, perspectives and conclusions

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Based upon concrete illustrations and key messages highlighted by the speakers, a discussion time will be open with the attendees in order to draw lines for a continuous improvement on the use of models in biomedical sciences. In an evolving context, where Replacement, Reduction and Refinement are more and more broadly embedded into animal protection regulations, it is of equal importance, for the benefits of both animals and science,



that scientists fully consider Rigor, Relevance and Reproducibility as their imperative drivers and work more openly across disciplines to bring their projects to success.

**Presentation:** Oral

892

## Developing performance-based qualification criteria for organs on a chip – EU perspective

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Regulatory testing of human medicinal products is carried out to support first administration to humans; before carrying out clinical trials in even larger populations; before marketing authorization or to control quality during production. Ethical and animal welfare considerations require that animal use is limited as much as possible. Directive 2010/63/EU on the protection of animals used for scientific purposes, unambiguously fosters the application of the principle of the 3Rs (Replacement, Reduction and Refinement) when considering choice of methods to be used.

The application of all 3Rs is currently embedded in the drafting process of regulatory guidance both at the European and at International Conference on Harmonisation ((V)ICH) level. With respect to non-clinical testing requirements for human medicinal products, over the past years, new *in vitro* methods have been accepted for regulatory use via multiple and flexible approaches, either as pivotal, supportive or as exploratory mechanistic studies, wherever applicable. Whilst replacement of animal studies remains the ultimate goal, the application of all 3Rs need to be the focus. As such, approaches aiming at reducing or refining animal studies are and have been routinely implemented in regulatory guidelines, where applicable.

This presentation will provide the regulator's view on the specific challenges and opportunities related to the regulatory acceptance of organ-on-a-chip technologies for the testing of human medicinal products. Briefly, the approach from the European Medicines Agency (EMA) to regulatory acceptance of 3Rs testing methods will be presented. In addition, the conclusions from the first EMA workshop on non-animal approaches in support of medicinal product development will be discussed and the experience gained from EU projects such as ORCHID, discussed. Focus will be drawn to the importance of context of use in the setting of qualification criteria, and the identification of reference compounds and performance standards.

**Presentation:** Oral

893

## A view from the veterinary sector on 3Rs in batch release

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Animal welfare and the 3Rs generally is a high priority across the veterinary medicines sector and has an impact across all stages of product development and supply from early-stage research through to routine product batch release. This talk will focus on the progress and strategies being made within and by the veterinary sector in relation to vaccine batch release as well as highlighting some of the challenges with global implementation. The sector is committed to developing and validating non-animal release tests for new products, but the biggest challenges exist with the older products, the talk will also highlight the positive progress being made through collaborations with EPAA, the HSI AFSA initiative and projects such as IMI2 VAC2VAC, bringing together stakeholders across the human and veterinary medicines sectors.

**Presentation:** Oral

894

## Examples of how data from *in vitro* developmental neurotoxicity assays could be used to make decisions about chemicals

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Over the past decade, a concerted effort has been made to develop *in vitro* and alternative assays that detect compounds with the potential to cause developmental neurotoxicity. As a result, a battery of assays has been developed that is believed to be useful for decision-making regarding developmental neurotoxicity (DNT) hazard associated with chemicals. However, case-studies that apply data from these assays to environmental decision-making have yet to be developed, in part due to sparse data on particular chemical(s) or chemical classes. Here, a series of hypothetical scenarios of how data from *in vitro* and alternative DNT assays could be used to inform decision-making regarding chemicals will be presented. These scenarios will range from simple prioritization of large numbers of compounds to more complex scenarios where both *in vitro* and *in vivo* data exist, and where exposure is also considered. Considering these theoretical scenarios is useful for two purposes. First, it can illustrate to deci-



sion-makers how data from these assays can be of utility to them. Second, and most important for scientists, it illustrates the guidance that the developers of these assays will need to provide to decision makers to facilitate their use of the data. Moving forward, these hypothetical examples will provide a framework to develop case-studies for real-life implementation of these assays (This abstract does not reflect EPA policy).

**Presentation:** Oral

895

## Role of biomedical journals' policies in promoting the dissemination of the 3Rs

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Animal-based research is not only regulated by legislation but also by self-regulatory mechanisms within the scientific community, which include biomedical journals' policies on animal use. They have a key role in promoting good principles and practice in animal research, since the motivation – and often the pressure – to publish may serve as an incentive for scientists to comply with journals' demands. Issues like quality of research and reporting, or the ethical treatment of animals, are particularly important for high-impact journals. Therefore, a re-evaluation of scientific journals' current policies, as well as an improvement in the communication between editors and reviewers to implement such policies is of huge importance to progress in the implementation of the 3Rs principles. A concerted approach, with sharing of information between editors, could be also strategic.

**Presentation:** Oral

896

## Microfabrication technologies for engineering of a moving joint-on-chip

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Osteoarthritis (OA) is a degenerative joint disease affecting more than 130,000,000 patients world-wide. The etiology of the disease is still poorly understood. While initially considered as a disease of cartilage it is now clear that the disease involves all tissues in the joint. A disease trigger in each of these tissues can initiate the onset of disease and each of these triggers converges over time in a similar disease presentation. Despite tremendous efforts in the recent past, the disease can still not be treated. It is believed that lack of translational power of currently used *in vitro* and *in vivo* models can at least in part explain this lack of success.

To address this caveat my group has started the development of a joint-on-chip. The joint-on-chip has a modular chip design (Piluso et al., 2019). We are engineering chips for individual tissue, i.e., cartilage, subchondral bone, synovium, ligament and/or meniscus. The individual chips are connected to each other through blood vessel mimicking microfluidic channels and with a chip mimicking the intra-articular space. This latter chip will contain features allowing non-invasive imaging/sensing of inter tissue communication. Since movement is a critical and essential feature in every joint, each chip can be actuated mimicking both compression and shear stress. Prototype chips of the synovium and the cartilage have become available (Paggi et al., 2020). Additionally, we developed various strategies for introducing cell-laden membranes composed of natural polymers and/or tissue constructs in the chip. Finally, we are developing sensors that can assess local matrix metalloproteinase activity, a key factor driving joint degeneration.

In my presentation, I will discuss various engineering aspects of our platforms. It is expected that these efforts will help us study the pathophysiology of osteoarthritis and will accelerate the development of dearly needed osteoarthritis disease-modifying treatments.

### References

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**Presentation:** Oral



897

## Complex *in-vitro* models: Synthetic matrices for pluripotent stem cells (PSC) derived multi-cellular 3D liver organoid

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Tissue-specific *in-vitro* organoid models have become a major technological breakthrough with tremendous potential. Most of the organoid cultures being developed using natural animal-derived ECMs making it difficult to precisely-define factors present in the microenvironment affect cells. We created a multi-cellular 3D liver organoid model completely defined synthetic matrices using polyethylene glycol (PEG), adhesion ligand peptides and MMP cleavable cross-linker based hydrogels. Multi-cellular human 3D liver organoids composed of PSCs-hepatocyte-, -stellate-, -endothelial and -macrophages-like cells exhibits some resemblance to *in vivo* derived liver tissue. An initial screen of > 200 microenvironments using PSC-hepatocyte progeny identified unique microenvironments that improved the hepatocyte phenotype and function by  $\pm$  50-fold over 2D culture. Next, we demonstrated that PSC-derived EC-, HSC- and -monocyte-like cells could also be maintained in the same microenvironment, in monoculture. When all four cell populations were incorporated into the hydrogels, complex spheroids were created. qRT-PCR and immunostainings demonstrated presence of multiple markers of all four cell types in the coculture for > 4 weeks. Functionality of hepatocyte-like cells, such as CYP3A4 activity, improved in the complex coculture over 2D/ or 3D monocultures. Studies are ongoing and will be presented that characterize the liver spheroids further, including the structural organization of the different cells, and their relative maturation, addressed by single cell RNAseq and multiplex immunostaining. In addition, data will be shown, demonstrating response of spheroids to profibrogenic and non-alcoholic fatty liver disease culture conditions. Thus, optimization of the cellular microenvironment that better mimics *in vivo* cell-cell interactions, mechanotransduction and chemical and cell adhesion cues found in primary liver, may enhance maturation of parenchymal and non-parenchymal liver cells generated from PSCs. Such PSC-derived multi-cellular human liver spheroids should be suitable for assessment of chemical toxicity, drug biotransformation and disease modeling that can be used by pharmaceutical industry in drug development pipelines.

**Presentation:** Oral

898

## Creating a culture that promotes replacement

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One of the main culprits in translational biomedical research is the lack of suitable predictive models. For our current understanding of the mechanisms that regulate disease, researchers have relied on biopsies from patients or on animal models. Patient biopsies provide a snapshot of the disease, usually in its final stages. However, biopsies do not provide information on underlying mechanisms or on temporal changes. For mechanistic studies, animal models are required as they take into account full physiological complexity. However, the problem with animal models is that the translatability to the human situation is poor. Respiratory system physiology, which is the main topic of study for me, is no exception to this problem. This is a major limitation, as the disease features that we face in the human pathophysiology might be completely different from what we observe in mouse models. This is a problem for the development of novel drugs that target the disease. Both this scientific need and the pressure from society demand the development of novel *in vitro* models that can replace animals. In the past two decades, several technologies have emerged which have proven to be highly suitable as human *in vitro* models, including human pluripotent stem cell (hPSC) technology, organs-on-a-chip (OoC) technology and organoid models. I will discuss the challenges researchers are faced with during a transformation from animal models to human *in vitro* models and provide a perspective on how to resolve these problems further in the future.

**Presentation:** Oral

899

## Increase predictivity and translatability from animal models – Understanding how different factors contribute to (ir)reproducibility

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Systematic and empirical assessments of biomedical research publications have proven useful in understanding the limits and impact of experimental design factors on reproducibility and translation of animal models. Much of this work has focussed on assessments of the methodological and reporting quality of



primary research to seek to understand the extent to which these studies are at risk of bias. Ideally, research reports include sufficient information to allow the reliability of the findings to be assessed, methodological details that describe how the study was done and allow the methods to be reproduced. Unfortunately, it is often the case that many studies either do not take or report measures to reduce risks of bias.

We have systematically collated data from over 7,000 studies describing 15,000 experiments covering 19 different disease models reporting outcome from nearly 200,000 animals.

Assessment of publication bias in 499 publications of focal ischaemia using 1300 animals identified that 1/6 experiments remain unpublished, which leads to an overstatement of the efficacy of at least 30%. Furthermore, only 3% of studies report performing a sample size calculation, and about a third of studies report random allocation to groups and blinded assessment of outcome – both associated with overstatements in reported efficacy. These findings are not unique to experimental stroke.

Fortunately, there are many resources available to researchers to improve the rigour of the design, conduct and reporting of studies. Improving the experimental validity of research will likely increase successful reproducibility and translation of animal models.

**Presentation:** Oral

900

## Communicating uncertainty about facts, numbers, and science in a polarized debate

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Uncertainty is an integral part of science and our knowledge about how the world works. Yet there can be anxiety among scientists and experts that communicating scientific uncertainty will only have negative consequences, such as signalling incompetence, encouraging critics and decreasing trust. This might be especially so in the context of a polarized discussion, in which it is feared that uncertainties can and will be misused. In this talk, I will discuss insights from (social) psychology about processes driving and reducing polarization in debates, and how uncertainties can be part of this. I will present a framework for communicating uncertainty about facts, numbers, and science, outlining the key aspects that should be considered for effective uncertainty communication. I will present the findings of recent empirical research examining the effects of uncertainty communication. My collaborators and I conducted four online experimental survey studies and one field study on the BBC News website to examine the effects of numerical and verbal uncertainty communication on people's trust in facts and numbers, and in the sci-

entists and experts producing those facts and numbers. Results show that whereas people do perceive greater uncertainty when it is communicated, there was only a small decrease in trust in numbers and in trustworthiness of the producers of these numbers, and mostly for verbal uncertainty communication. Together, these insights and findings could help communicators of facts and science on alternative and animal use in the life sciences avoid polarization and be more open and transparent about the limits of scientific knowledge.

**Presentation:** Oral

901

## Functionally enigmatic genes in cancer: Are we still looking under the lamp post for the keys?

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Despite cancer being a comparatively well-studied disease that has benefited from decades of intense focus, we have recently demonstrated that a substantial number of genes implicated in cancer are relatively poorly studied, and that these genes are systematically overlooked by any data analysis pipeline, such as enrichment analysis, that depends on database annotations for understanding biological function. Moreover, these genes are not missing at random but reflect that our information about genes is gathered in a biased manner: poorly studied genes are more likely to be primate-specific (which has important consequences for clinical research), less likely to have a Mendelian inheritance pattern, and they tend to cluster in some biological processes (such as mRNA splicing) and not others (such as cell-cycle). Although this is likely the result of both technological limitations as well as the fact that well-known genes tend to gather more interest from the research community, in the absence of a concerted effort to study genes in an unbiased way that is not dependent on annotations, many genes (and biological processes) will remain opaque – we will in essence continue to be looking under the lamp post for the lost keys, because that is where the light is.

**Presentation:** Oral



902

## Reference-free annotation for single-cell transcriptomics using graph neural network model

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Recent advances in single-cell RNA sequencing (scRNA-seq) have furthered the simultaneous classification of thousands of cells in a single assay based on transcriptome profiling, wherein accurate cell-type annotation is critical for mechanistic studies including risk and safety assessment. In most analysis protocols, cell type annotation of single cells is performed heavily based on the reference of cell marker genes or RNA-seq profiles, resulting in the poor expansion of these methods. Herein, we introduce CellSort, which is reference-free annotation of single cells based on a deep learning model of graph neural network (GNN). Using three scRNA-seq data resources of human, mouse and *C. elegans* atlases, the feasibility of CellSort were demonstrated by high concordance among 816,505 cells across three species involving 56 human tissues and 31 mouse tissues. Furthermore, CellSort outperformed other reference-dependent methods on annotating another 85 independent scRNA-seq datasets including 225,370 cells from 11 human tissues and 144,527 cells from 13 mouse tissues. The present results showed CellSort accurately revealed cell identities without prior knowledge of marker genes or RNA-seq profiles, thus potentially providing new insights into mechanisms underlying disease pathogenesis and progression at a single-cell resolution.

**Presentation:** Oral

905

## A novel 3D hepatic bioprinted model

*Eleonore Attignon<sup>1</sup>, Fabien Guillemot<sup>2</sup>, Laurent Rutault<sup>1</sup>, Françoise Goldfain-Blanc<sup>1</sup> and Hélène Aerts<sup>1</sup>*

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Drug-induced liver injury (DILI) is a major concern for the pharmaceutical industry. Reliable prediction of DILI in preclinical stages is difficult; this is why it is essential to improve tools used in predictive toxicology. Three-dimensional (3D) cell printing technology has emerged as an innovative technology. The objective of this project is to establish and characterize a novel bioprinted human liver tissue in a defined architecture in order to mimic *in vivo* liver.

Poietis' Laser Assisted Bioprinted (LAB) technology is used to construct a 3D liver model composed by the differentiated hepatic cell line and non parenchymal cells (including Stellates

cells and Kupffer cells). Cell viability was analyzed by live/dead staining and LDH quantification in the supernatant. For characterization purposes, the bioprinted hepatic model structure was analyzed by Hematoxylin Eosin Saffron (HES) and Periodic Acid-Schiff (PAS) coloration. Hepatocyte functionality was evaluated by albumin secretion and by immunohistochemistry using specific hepatic markers.

The first results showed that bioprinted cells are viable and express specific markers over time culture, CD68 for human Kupffer primary cells; vimentin for quiescent human Stellate primary cells and HNF4 alpha, CYP3A4, albumin and CK19 for differentiated HepaRG cell line. PAS staining show that bioprinted cells are capable of glycogen storage.

The 3D bioprinted hepatic model shows functional properties. The characterization of this 3D bioprinted model is still in progress and this engineered human liver model will be useful to understand hepatotoxicity mechanisms of drug substances and will detect, as early as possible, any adverse effects when developing medicinal products.

**Presentation:** Oral

906

## Deep learning for predicting molecular properties

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In recent years there has been a growing interest in the application of deep learning across chemical space to predict properties of molecules (e.g., bioactivity, solubility) or to design molecules and materials with desired properties. In this talk we show, (i) how deep neural networks can learn an information-rich molecular feature representation from low-level encodings of a huge corpus of chemical structures (Winter et al., 2019), (ii) how this representation can be extracted for each new molecule and used as a descriptor for quantitative structure-activity relationships (QSAR tasks) and (iii) how a set of different physico-chemical ADMET endpoints (e.g., logD in neutral and acidic pH, solubility melting point, membrane affinity, and human serum albumin binding) can be combined in a single multitask graph convolutional regression model which thereby achieves increased predictive power compared to single-task regression models and previous modelling methods (Montanari et al., 2020).

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**Presentation:** Oral

907

## Recombinant antibodies: A complete toolbox for academia

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There are many reasons to favor the use of recombinant antibodies, but the tools available to academic researchers have been limited until recently. It used to be a significant effort to even know when a recombinant existed against your favorite protein, what its main characteristics were, or how it should be used. It was expensive and difficult to develop new recombinant antibodies by phage display, to sequence hybridomas, or even simply to produce an existing recombinant antibody. The University of Geneva has developed an open non-profit academic resource that overcomes these hurdles. The ABCD database provides an open-access comprehensive list of most existing recombinant antibodies linked to their antigenic target. An academic production facility linked to the database allows to gain access to any sequenced antibody at a very low price. Antibody Reports is an open-access scientific journal dedicated to characterization of recombinant antibodies. A discovery platform proposes to discover new antibodies, or to sequence previously selected hybridomas. This collective enterprise will make it possible for the entire scientific community to switch to animal-free, well-characterized, sustainable, and affordable research reagents.

**Presentation:** Oral

908

## Buiding vessels on a chip to model genetic vascular diseases using patient-specific induced pluripotent stem cells

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Blood vessels are integral to the maintenance of all tissues. They deliver oxygen and nutrients and remove waste. Blood vessels not only contain endothelial cells (ECs) that are in direct contact

with flowing blood cells and fluids but they may also have pericytes and smooth muscle cells on the outside that stabilize and regulate their size. Defects in EC and pericyte/smooth muscle cell interaction are often implicated in many pathological conditions. Patients with a genetic disease called hereditary hemorrhagic telangiectasia (HHT) suffer from heavy and recurrent nosebleeds, due to unstable blood vessels as a result of defective interaction between ECs and pericytes. Here we used human induced pluripotent stem cells (hiPSCs) as a source of patient-specific cells, as they can be derived from all individuals, including children and patients with genetic disease and subsequently differentiated towards all cells of the body. Besides, we can now use hiPSCs to derive all the component of blood vessels in large numbers and use them to re-create blood vessels on a chip to model inflammation and disease. HHT-hiPSC-ECs displayed no apparent functional differences using a set of standard two-dimensional (2D) assays. The ability of hiPSC-ECs to form a perfused microvascular network was next examined using three-dimensional (3D) cultures in microfluidic chips. Using these 3D vessels on a chip, we found that in contrast to 2D microvascular cultures, the ability to form 3D microvessels in microfluidic chips was strikingly compromised when HHT-hiPSC-ECs were used compared to isogenic control ECs. This patient-based hiPSC model thus serves as the first proof of principle that vascular diseases could be modeled using patient-specific hiPSCs in 3D microfluidic chips and to identify new target cells and possible pathways for therapy.

**Presentation:** Oral

909

## Canadian Centre for Alternatives to Animal Methods

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The first and only Canadian Centre for Alternatives to Animal Methods (CCAAM), and its subsidiary, the Canadian Centre for the Validation of Alternative Methods (CaCVAM) were officially launched at the University of Windsor in Ontario, Canada in October 2017. The centre's research and training laboratories, the Eric S. Margolis Research and Training Laboratories for Alternatives to Animal Methods, were officially established in October 2019. The overarching vision of CCAAM is to promote the replacement of animals in Canadian biomedical research, education, and regulatory testing through 21<sup>st</sup> century science, innovation, and ethics. CCAAM/CaCVAM serves as the leader and nexus to bring together national academic, industry, government, and public stakeholders to develop, validate, and promote the acceptance of new approach methodologies to replace the use of animals in science in Canada. This presentation will cover our multifaceted mission executed mainly through three unique pil-



lars: Research Pillar (to develop, validate, and promote non-animal methods for hypothesis driven basic biomedical research); Academic Pillar (academic programs to train the next generation to think outside the cage); and Regulatory Pillar (to modernize chemical safety testing in Canada and accelerate the acceptance of non-animal methods by working closely with Canadian regulators, especially Health Canada). Through our multidisciplinary work, we are contributing to global 3Rs efforts in a uniquely Canadian way – to promote a paradigm shift in which human biology serves as the gold standard.

**Presentation:** Oral

910

## Beyond animal research: Human biology as the gold standard

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One of the greatest challenges facing modern biomedical research is understanding the molecular mechanisms underlying complex, multifactorial diseases such as cancer, heart failure, neurodegenerative disorders, diabetes, stroke, and infectious diseases, among many others. It has become evident that overreliance on animal models has hindered the development of disease-modifying therapeutics across the fields. It is undeniable that a new approach is warranted, a new direction with *Homo sapiens* as the gold standard animal model where integrative human biology-based experimental approaches are unquestionably indispensable. Transforming a research culture where mouse data take precedence over human data to accept humans as the quintessential animal model is no easy feat. This will require a paradigm shift in every aspect from technological advancements to overhauling longstanding practices ingrained in the current research culture. This presentation will outline multifaceted strategic areas addressing key needs, opportunities, challenges, and barriers to implementing research directions – based on human biology in health and disease – enabling discovery science that makes the human a better experimental model to accelerate translation. The primary goal is to promote interactive discussion with the audience to collate recommendations for a “Predictive Biology Roadmap” to prioritize human biology as the gold standard for 21<sup>st</sup> century biomedical research.

**Presentation:** Oral

911

## Novel flame retardants and hepatic steatosis: Elucidation of mechanisms to develop (quantitative) adverse outcome pathways, (Q)AOPS

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Flame retardants are chemicals or mixtures widely used in commercial and consumer products to reduce flammability. Following the ban of long used polybrominated diphenyl ethers, a wide range of novel flame retardants (nFRs) used as a replacement are consistently found at relatively high levels in the environment and human matrices. Some evidence indicates the toxic effects of individual nFRs in mammals, but generally, toxicity data remains insufficient. Considering the widespread presence of these compounds, this is a crucial knowledge gap, and more toxicological studies on nFRs, individually and as mixtures are critically needed (Bajard et al., 2019). In line with the 3Rs (reduction, refinement, and replacement) principle of regulatory toxicology, *in vitro* approaches combined with the Adverse Outcome Pathways (AOPs; an OECD endorsed framework) allow to link mechanistic studies to apical endpoints.

The present study aims to better understand the mechanisms driving the liver toxicity of prioritized nFRs, focusing on hepatic steatosis, and to contribute to the development of quantitative AOP (qAOP). For steatosis, AOPs and AOP network linking several molecular initiating events (MIEs) have been suggested. Evidence indicates that the prioritized nFRs affect some relevant MIEs. Experimental research is being conducted to quantify the effects of the nFRs on different Key Events of the AOPs proposed for hepatic steatosis, using *in vitro* toxicological studies in 2D and 3D cell culture. Our first results indicate that several nFRs may induce lipid accumulation in the hepatocytes at sub-cytotoxic concentrations. Additional *in vitro* test battery, such as nuclear receptor activation, expression of associated downstream proteins, are currently being examined to understand the molecular mechanism and to develop quantitative data for follow-up development of qAOP using the computer simulations through Artificial Intelligence-based tools. Potential mixture effects of nFRs on the studied endpoints are being assessed as well, and relevant AOP networks will be discussed.

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**Presentation:** Oral



912

## Regulatory acceptance for the substitution of *in vitro* for *in vivo* vaccine potency and safety assays for batch release: Science versus the fear factor

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**Introduction:** Vaccine quality control (QC) strategies for new products typically involve physical chemical methods, *in vitro* assays, QPCR and NMR. However, for most legacy vaccines (e.g., DTaP, rabies virus vaccines etc.) *in vivo* potency and safety methods predominate. What has prevented implementation of innovative, more robust, effective and stability indicating *in vitro* assays for legacy vaccines, relative modern vaccines and biotherapeutics?

**Issues:** Challenges preventing one-to-one *in vivo* assay replacement with superior *in vitro* alternatives include:

- i. high variability of some animal methods prevents the establishment of a correlation between the existing and alternative assay due to the performance of the *in vivo* assay, not the *in vitro* alternative,
- ii. detailed characterization of legacy products with modern methods, needed define the critical quality attributes of the antigen(s) and assay reagents (e.g., mAbs) that could be used *in vitro* methods,
- iii. unwillingness of regulators to put aside long held false assumptions regarding the performance and value of animal assays.

**Relevant guidance:** To support the implementation of *in vitro* methods, the European Pharmacopoeia has developed a General Chapter entitled, Substitution Of In Vivo Method(s) By In Vitro Method(s) For the Quality Control Of Vaccines, (Ph. Eur. 5.2.14). This guidance proposes a new approach referred to as substitution, where one-to-one replacement has not been feasible or is scientifically unjustified. The guidance also provides a critical assessment noting that animal assays are less suitable QC methods relative to appropriately designed *in vitro* assays.

**Conclusion:** This new regulatory perspective has provided support for industries to invest in *in vitro* assay development. It has also accelerated the discontinuation of longstanding animal-based tests, which are now understood to be scientifically unjustified. Two examples of the latter are the recent discontinuation of the General Safety Test and the Histamine Sensitization Test from the Ph. Eur.

**Presentation:** Oral

914

## Finding alternatives using text-based article classification

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The implementation of 3R principles in ongoing research is hindered by a number of factors. One of the main challenges is to find valid and accurate information on the possibilities for all the 3Rs in general, and in Replacement in particular. It has been shown that finding replacement alternatives in the literature is an extremely challenging task.

To facilitate the implementation of the 3Rs, we have developed an algorithm that uses a combination of text mining and artificial intelligence tool for rapid and effective searches for identifying Replacement alternatives in the entire MEDLINE database. The model has been trained on a set of known, trusted papers that describe alternatives from a variety of research fields and is thus suitable to address a wide range of research questions.

In this talk we will present the predictive algorithm as well as a software application that incorporates this algorithm in the context of a large database of > 200,000 biological concepts and a graphical user interface. As such the algorithm can be accessed over the internet from any device. Results from this algorithm can be exported to all major reference managers for use in grant proposals, research papers and systematic or scoping reviews.

The software is freely available for academic use and educational purposes.

**Presentation:** Oral

915

## A funder's role in stimulating transparency in 3Rs research

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The Netherlands Organisation for Health Research and Development (ZonMw) aims with the More Knowledge with Fewer Animals program, to promote development and implementation of animal free innovations in health research, without impairing quality of scientific research and safety of products. The program is divided into different topics and seeks collaboration with research institutions and private partners.



Here, we focus on the topics within “The knowledge infrastructure module”, which is as of 2012 dedicated to increasing value and transparency of animal studies in three ways. First, it offers small grants for research institutions allowing them to organize “in house” systematic review (SR) workshops, for animal studies. The one-day workshops can be attended by twenty of their researchers. Second, it offers small grants to allow researchers to conduct a SR for animal studies. These grants provide for FTE (two-months) and counselling, to guarantee the quality of the SRs. Third, it promotes open access publication of negative & neutral results of animal experiments by compensating employment costs (one-month). Grantees are obliged to publish these articles adhering to the ARRIVE guidelines and to register the studies at an online register for animal research, Preclinicaltrials.eu. The workshops and the counselling of a SR are supervised by SR qualified researchers (Radboudumc), in collaboration with ZonMw. ZonMw is looking into ways to monitor these outcomes in an automated manner, through machine readable grant-associated metadata. Based on these techniques, we also explore the possibility to improve the findability of animal free innovations in literature.

Eventually, these strategies will impact the value and transparency of research output, helps to avoid unnecessary repetition and to reduce the bias in the current literature. This strategy fits well in the transition to Open Science, more specifically by stimulating transparent and responsible animal research (see also abstract #351).

**Presentation:** Oral

916

## Data management in a changing toxicity testing paradigm

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Skills4science is an initiative intended to provide early-stage researchers in toxicology and beyond with skills to successfully navigate the intricacies of a life science career. Data management is one such skill that is increasingly recognised as a corner stone of good science. Specifically, toxicology research is becoming increasingly data-intensive, both in terms of volume and complexity. This is largely due to the advent of exciting developments integrating new approach methodologies to replace, refine and reduce traditional animal testing. These methodologies include “omics” technologies and an ensemble of machine learning and deep learning approaches for establishing (quantitative) structure activity relationships. These approaches come with avalanches of data which makes making the data findable, accessible, interoperable and reusable (FAIR) challenging. Here, a number of data management plan writing

initiatives from academia and beyond and their applicability to toxicology are discussed.

**Presentation:** Oral

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## Deciding on an assay setup to control test chemical concentrations *in vitro*

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The nominal concentration, i.e., the theoretical concentration based on amount of test chemical added to culture medium, is generally used to express concentration-effect relationships in *in vitro* toxicity tests. However, for unstable, volatile, lipophilic and highly plasma protein bound chemicals, the nominal concentration does not represent the concentration responsible for the observed effect at the target site in cells. Within the Create2Solve project, we develop tools that control for the degradation, evaporation and binding of chemicals to the *in vitro* system setup of these chemicals. We compare concentration-cytotoxic response relationships of “difficult-to-test” chemicals, including alkylbenzenes, in sealed glass vials, 3D printed non-binding test materials, and microtiter plates dosed through polymers loaded with test chemicals (i.e., partition-controlled dosing). A decision tree is evaluated to allow toxicologists to determine whether and when the use of each of these tools is necessary and applicable for the chemical and *in vitro* biomarker they wish to test.

**Presentation:** Oral

918

## Comparing model predictions and analytically determined test chemical distributions *in vitro*

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The absorption, distribution, metabolism and excretion of chemicals (ADME) play a central role in quantitative *in vitro in vivo* extrapolation (QIVIVE) studies, as these processes determine the concentration of the chemical at the target organ where the toxic effect is initiated *in vivo*. Although largely ignored, similar kinetic processes determine the target concentration and thus the level of bioactivity of chemicals in *in vitro* assays. Despite chemicals or assays eliciting similar effects at similar nominal concentrations, the bioavailable concentration may vary greatly between chemicals and assays, thus hampering comparisons. A number



of partition models have been developed to estimate target concentrations of a chemical *in vitro*. The aim of this study is to review these models in terms of their chemical and assay applicability domains, inclusion of kinetic processes, input parameters, and the extent to which they have been evaluated. Subsequently, model predictions of the distribution are compared to measured time-resolved concentrations of these chemicals associated to well plate plastic, exposure medium, cells and cell attachment matrices in intrinsic clearance and cytotoxicity assays with cells cultured in suspension, monolayers, sandwich and spheroids. As a result of the variation in phases and kinetic processes included in the partition models, free concentrations vary significantly for the lipophilic, quickly cleared chemicals between models and between simulated and analytically measured concentrations.

**Presentation:** Oral

919

## Using human to bench to human circular approaches for health and medicines research

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Last decade sea of science/technology progress is deeply changing society functioning, including health/disease management, medicines development. Animal-based research has been gold-standard on disease/medicines research (e.g., disease models, genetically modified animals) also for regulatory purposes. But animal to human predictions are limited, and alternative/complementary approaches are needed. Deeply interconnected, science and technology are providing human-based innovative tools based as those using human iPSC technology, for disease research, human associated-targets, or investigational molecules as potential therapies. Human cells are increasingly used for (dysfunctional) target identification in health/disease conditions. Information is used by computational chemistry and biology to conceive and develop corrective molecules/approaches. Patient's variability on disease expression, progression, response to therapies is identified from big data captured by health systems/clinical research and provide basis for patient and disease stratification. Specific patient-derived samples are used to identify molecular/genetic attributes behind variabilities, providing the basis for conceiving more adjusted, personalised treatments. Therefore, medicines research can increasingly include 1. patient's samples selected based on specific attributes, 2. identifying dysfunctional targets 3. computational conception of target molecules screened *in vitro*, *in silico* to select the most promising/safe candidates 4. testing those in human-based cell/organ systems, anticipating efficacy and potential safety hurdles, replacing animal

models, hopefully towards their elimination. Those should enable progression into carefully designed human studies, incorporating safety/efficacy biomarkers and digital tools for events reporting and patient monitoring. Identification of responders/non-responders/adverse reactions can be achieved using those tools, providing foundation for another wave of research using patient's samples to enable again identification of targets responsible for those variabilities and search for solutions. Successive waves of circular, continuous research, from patient to bench to patient will hopefully result in tailored treatments towards personalised medicine.

**Presentation:** Oral

920

## Investigating epilepsy using a combination of mathematical modelling and voluntary human data as a viable replacement for animal models

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Epilepsy is characterised by abnormal electrical brain activity (seizures) that vary considerably between individuals. Both diagnosis and treatment of epilepsy are highly patient specific. The aim here is to understand patient specificity by studying individual brain structure-function relationships. We investigate the relationship between a patient's brain structure (connectivity) and dysfunction (seizures), which is a fundamental problem of 21<sup>st</sup> century epilepsy research.

The overwhelming majority of neuroscience research is based on animal experiments. Animal models (typically mice & rats) are used to test hypotheses by artificially inducing epilepsy by administration of drugs, electrical stimulation, and by breeding genetically modified animals that produce seizures. Whole brain or brain slices of animals are used to study the relationship between brain connectivity and seizures. Since brain connectivity is unique for each individual animal, experiments are limited specifically to each animal. We propose an alternative to animal models as a combination of voluntary human data and a mathematical model of brain connectivity and epileptic behaviour.

Although a computational model is not as detailed as an actual physical brain, the model parameters are rigorously understood and can be manipulated at will, unlike a physical experiment. Within this framework we can modify many aspects of brain connectivity with ease and study its effect on seizure dynamics. This work moves away from traditional neuroscience paradigms and replaces animal models with computational models based on



human data. We are interested in how the brain connectivity of individual patients' influences the transition from a normal state into an abnormal seizure state. This novel multidisciplinary approach in combination with voluntary human data, will serve as a replacement for animal models and will contribute to the paradigm shift away from conventional neuroscience research.

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**Presentation:** Oral

931

## A 3D autologous iPSC-derived hair bulb model

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Human hair follicles are complex skin appendices dependent of epithelial-mesenchymal crosstalk. The arrangement of the hair bulb keratinocytes and melanocytes surrounding the dermal papilla is essential for the hair shaft production and melanisation. Currently, most research on the hair follicle focuses on animal models. Although several factors seem similar, there are many differences between the physiology of human hair follicles and those of animal.

In the present work, we are developing a 3D autologous hair bulb model based on induced pluripotent stem cell (iPSCs). These iPSCs are on the one hand differentiated into hair bulb keratinocytes by stimulation of the ectodermal lineage. In addition, after an induction into neural crest cells, a specialisation for maturation of melanocytes and dermal papilla cells is generated. We optimized the differentiation protocols for all three cell types and confirmed their functionalities, e.g., by the expression of bulb markers (K14, MSX1, MSX2, DLX2, RGS2, TP63) for the iPSC-derived keratinocytes and the validation of the activation of specific melanocyte pathways (MITF, TYR, TRP1, TRP2, SOX10), melanogenesis and melanin transfer for the iPSC-derived melanocytes. Concerning the dermal papilla cells, we confirmed comparable expression profiles to primary cells (versican, CORIN, vimentin, NESTIN, LEF1).

After differentiation, the three cell types are combined into an autologous hair bulb model. We confirmed the hair bulb structure and a partially restored crosstalk (Wnt, BMP, PDGF) between iPSC-derived keratinocytes and iPSC-derived dermal papilla cells.

The next steps will be the validation of this model by treatment with pharmacologic molecules targeting hair follicles.

**Presentation:** Oral

935

## Application of the 3Rs in creation of GAA mice – The challenges of new technologies

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Although animal welfare is a growing societal concern, we have not seen a significant decrease in the total number of animals used in animal experiments in the last years. In particular, the number of genetically modified mice used in experiments remains high. Since the amazing discovery in 2012 that the endonuclease system CRISPR/Cas9 can be used very efficiently for gene editing, a revolution in genetic engineering has taken place. Among mammals, the mouse remains the predominant experimental animal because of its obvious advantages in size and reproductive speed. The revolution triggered by CRISPR/Cas9 molecular scissors increasingly influences the transgenic field and raises new questions, at the same time opening up new possibilities and disappointing expectations. An impact on the 3Rs is evident. For example, we must ask ourselves what the risks are that more and more animals will be used simply because of the availability of new techniques. In addition, additional species become amenable to genetic alteration resulting in new ethical challenges. On the other hand, we need to investigate the possibilities of making a positive contribution to the 3Rs by using modern techniques of genetic modification without categorically excluding well-established ones. Given the current state of research, the potential is great. There is the possibility of positively influencing the balance between the burden as well as the number of animals used and the quality as well as quantity of the knowledge gained.

**Presentation:** Oral



936

## Human organ chips: From experimental models to clinical mimicry

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This presentation will highlight advances that my team at the Wyss Institute has made in the engineering of human “Organ-on-a-Chip” (Organ Chip) microfluidic cultured devices lined by living human cells that recapitulate organ-level functions as a way to replace animal testing for drug development, mechanistic discovery, and personalized medicine. I will describe the engineering of multiple human organ chips, including lung, intestine, kidney, bone marrow, liver, lymph node, and coupled blood-brain barrier and brain neuronal network chips. By recreating organ-specific physical and chemical microenvironments, including fluid flow, mechanical motions, oxygen gradients, and the presence of complex living microbiome, we obtain a level of biomimicry of organ-level functions not possible in other *in vitro* models. These Organ Chip models also have been adapted to develop multiple human disease models (e.g., pulmonary edema, asthma, COPD, influenza, colitis, environmental enteric dysfunction, lung cancer, esophageal cancer, radiation toxicity, and rare bone marrow disorders), identify approved drugs that might potentially be repurposed as COVID19 therapies, and discover new therapeutics. A Bone Marrow Chip was recently developed that precisely mimics human bone marrow toxicities induced by clinically relevant (pharmacokinetic) exposures to drugs as well as by radiation exposure, and it also was used to create personalized models of a rare genetic disorder of the marrow that provided new mechanistic insight into this disease (Chou et al., 2020). In addition, we engineered rat, dog, and human Liver Chips that recapitulate species-specific drug hepatotoxicities, which could help to replace use of these animals in preclinical drug development (Jang et al., 2019). Finally, I will describe how we have integrated multiple Organ Chips into an automated “Human Body-on-Chips” that enables real-time analysis of cellular responses to pharmaceuticals, chemicals, radiation, and toxins, as well as quantitative *in vitro*-to-*in vivo* extrapolation of human drug pharmacokinetics and pharmacodynamics (Herland et al., 2020).

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**Presentation:** Oral

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## Vaccine batch to vaccine batch comparison by consistency testing (VAC2VAC)

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VAC2VAC brings together a unique One Health consortium of pharmaceutical companies, academia, translational research organisations, Official Medicines Control Laboratories and regulatory bodies with the overall objective to demonstrate proof of concept of the consistency approach for batch release testing of established vaccines. This means that non-animal assays – instead of animal tests – can be used to ensure that each vaccine batch produced is consistent with a batch already proven to be safe and efficacious. It covers vaccine potency, safety and animal welfare.

The three main steps to reach these objectives are:

- 1) Development of new or optimisation of existing non-animal methods for consistency testing: The core activity of the project, focuses on development and optimisation of physico-chemical methods, immunochemical methods, cell-based assays, and multi-parametric assays & bioinformatics.
- 2) Pre-validation of selected methods: For selected methods developed in VAC2VAC, small-scale multi-centre studies will be set up to assess transferability and inter-laboratory reproducibility of the methods. Methods that are successful and are proposed for inclusion in Eur.PH., will be submitted to the EDQM Biological Standardisation Programme to be considered for further validation studies.
- 3) Regulatory acceptance of the consistency approach: To maximise the chances of global acceptance and regulatory acceptance of the consistency approach, a close interaction between the consortium with regulators will be put in place.

The presentation will outline the project in detail and give an update on the status of the different assays under development.

*Consortium:* EVI; RIVM; NIBSC; PEI; JRC, UU, UMCG; Intravacc; HU; ISS; AGES; IABS-EU Sciensano; BPRC; Sanofi; Zoetis; Merial; Boehringer Ingelheim; MSD; GSK; MEB; Pfizer.

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**Presentation:** Oral



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## The in3 project – An integrated interdisciplinary approach to animal-free nanomaterial and chemical safety assessment

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The in3 project (<https://www.estiv.org/in3/>) is funded by the EU's Marie Skłodowska-Curie Action – Innovative Training Network (MSCA-ITN for short) that aimed to drive the synergistic development and utilisation of *in vitro* and *in silico* tools for human chemical and nanomaterial (NM) safety assessment. The project focused on differentiation of human induced Pluripotent Stem Cells (hiPSC) to toxicologically relevant target tissues including brain, lung, liver and kidney. The tissues, from the same genetic backgrounds, were exposed to common compounds and the data generated and prediction tools generated will be used to develop modernised safety assessment approaches combining cheminformatics, mechanistic toxicology and biokinetics into computational models which can account for donor and tissues specific effects. The project employed 15 PhD students to carry out these activities in a coordinated and collaborative fashion. The project objectives and outcomes will be discussed.

**Presentation:** Oral

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## A human iPSC-based microphysiological model of the liver to study the impact of hepatic stellate cells on NASH development

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**Introduction:** In developed countries, non-alcoholic fatty liver disease (NAFLD) is increasingly the major form of chronic liver disease. This is related to risk factors such as a fatty diet, hypertension, smoking, genetic background and intestinal dysbiosis (Huang et al., 2019; Benedict et al., 2017). In some cases, hepatic steatosis progresses to non-alcoholic steatohepatitis (NASH)

which in time can lead to liver cirrhosis and to hepatocellular carcinoma. NASH symptoms are often silent until late stages of the disease making the identification and treatment of patients difficult. One major contributor are hepatic stellate cells (HSCs) which become activated, differentiate into myofibroblast-like cells and start to secrete inflammatory cytokines as well as to increasingly deposit extracellular matrix proteins such as fibronectin and various collagen forms. Here, we present the development of a human iPSC-based microphysiological liver model to study the interplay between HSCs and other liver cell types in the context of NASH. Our aim is to provide a tool for future mechanistic studies during the development of treatment options.

**Theory and Experimental procedure:** Human iPSCs have been differentiated into iPSC-derived endothelial cells (ECs), iPSC-derived hepatocytes and iPSC-derived HSCs (Orlova et al., 2014; Kajiwara et al., 2012; Peters et al., 2016). Further, each cell type has been characterized for cellular morphology, cell type-specific marker expression and key functionality in single cell cultures before integrating them as a triple coculture into a biochip. Additionally, inflammatory conditions and a fatty acid rich diet were investigated to assess the relevance of the iPSC-derived cells contributing to a disease model of NASH.

**Results and Discussion:** The differentiation of iPSC-derived ECs, hepatocytes and HSCs could be successfully adapted. iPSC-derived hepatocytes show CYP3A4, ASGPR-1, E-cadherin and MRP-2 expression, apoB, albumin and collagen I and IV production. Further a strategy for the seeding of iPSC-derived hepatocytes into the biochip could be identified. iPSC-derived ECs were found to be positive for CD31, von Willebrand factor, VE-Cadherin, ZO-1,  $\beta$ Catenin and only low levels of ICAM-1 and VCAM-1. They functionally responded with increased ICAM-1 and VCAM-1 levels to stimulations with TNF as well as fatty acids. iPSC-derived HSCs were characterized for PDGFR $\beta$ , collagen I, collagen 1 $\alpha$ 1, vimentin and  $\alpha$ SMA expression. Further they showed superior vitaminA storage capacity compared to standard HSC cell lines. Functional responses to inflammatory triggers as well as fatty acid rich diets are under current investigation. First results indicate the functional capacity for increased collagen 1 $\alpha$ 1 expression and increased collagen deposition.

**Conclusion:** Here we present a promising strategy to develop an iPSC-derived microphysiological model of the liver to study NASH. A functional relevant human model of this disease is of high importance as quiescent and activated HSCs behave differently in humans compared to disease models established with rodents. Further, the definition of biomarkers, early disease mechanisms and potential treatment options is relying on comprehensive *in vitro* models based on relevant human physiology.

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**Presentation:** Oral

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## Chinese perspective of the implementation of organ-on-chip-based assays into the regulatory landscape

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Microphysiological systems (MPS), microfluidic devices capable of emulating entire organismal functionality, have the potential to serve as a new enabling platform to predict the efficacy, toxicity and pharmacokinetics of drug compounds in humans. Many academic research institutions in China currently have independently developed a variety of organ chips, including 3D microvascular models, cardiac chips and blood-brain barrier systems. However, the development of MPS in China still remains in the academic research stage and has not yet reached industrialization. The MPS-based test analysis is also limited to laboratory research; it has neither been adopted by the pharmaceutical industry yet nor entered the regulatory evaluation process. The National Institutes for Food and Drug Control (NIFDC) is a subordinate agency of the National Medical Products Administration (NMPA). The NIFDC has cooperated internationally regarding regulatory science in the field of “Human-on-a-chip” technologies with the Technical University Berlin, Germany, to get hands-on experience with MPS-based approaches to reduce regulatory barriers for acceptance of the emerging technology. The NIFDC team is conducting toxicology endpoint-driven experiments for a human two-organ arrangement on the chip. More tests will be carried out in the field of toxicity and efficacy evaluation using multi-organ chips. The Chinese NMPA will improve the interactions among regulators on a global basis to move emerging sciences and technologies forward concerning the use of MPS data and its acceptance for regulatory decision-making.

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**Presentation:** Oral

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## Gastruloids from stem cells: Models of early development

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Gastruloids are three-dimensional aggregates of embryonic stem cells that display key features of mammalian development after implantation, including germ-layer specification and axial organization. We have shown that gastruloids can be developed to fairly advanced stages from mouse, but this is somewhat less advanced using human cells. Nevertheless, these models show the power of gastruloids as a model system for exploring development and somitogenesis *in vitro* in a high-throughput manner.

**Presentation:** Oral

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## Human pluripotent stem cell models for cardiotoxicity

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Animal models are 78% accurate in determining whether drugs will alter contractility of the human heart. To evaluate the suitability of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) for predictive safety pharmacology, we quantified changes in contractility, voltage and/or Ca<sup>2+</sup> handling in 2D monolayers or 3D engineered heart tissues (EHTs). Protocols were unified via a drug training set, allowing subsequent blinded multi-centre evaluation of drugs with known positive, negative or neutral inotropic effects. Test conditions were refined



by adopting approaches to reduce signal-to-noise ratio, reduce spontaneous beat rate or enable chronic testing, improving accuracy to 85% for monolayers and 93% for EHTs. Contraction amplitude was a good predictor of negative inotropes across all the platform-cell configurations and of positive inotropes in the 3D EHTs. Of the platform-cell configurations, responses in EHTs aligned most closely to the free therapeutic plasma concentration. This work will be discussed in more detail on the context of animal alternatives for predicting cardiac toxicity.

**Presentation:** Oral

946

## Developing performance-based qualification criteria for organs on a chip – US FDA perspective

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*In vitro* Microphysiological Systems (Organs on a Chip or MPS) technology has the potential to address many of the shortfalls of *in vivo* animal testing, 2D *in vitro* testing and even some of the issues with other 3D *in vitro* models. Although there has been extensive global development of MPS models, there has been a lack of qualification criteria for any of these devices for use in either medical or non-medical regulatory applications. Global agreement between all sectors on performance-based criteria for acceptance of MPS data is essential to move this technology into the regulatory arena. The establishment of a forum for global regulators from all product sectors to discuss these issues is the most effective way to accelerate the introduction of any new disruptive innovation into the regulatory paradigm. It allows global scientists to work in an environment that fosters creative thinking and promotes scientific multidisciplinary interaction and collaboration.

**Presentation:** Oral

947

## Science, ethics and acceptance of human microphysiological systems – An ultimate alternative to testing in laboratory animals and human volunteers

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Microfluidic microphysiological systems (MPS) have proven to be a powerful tool for recreating human tissue- and organ-like functions at research level. This provides the basis for the establishment of qualified preclinical assays with improved predictive power. Reduction and replacement of laboratory animals and healthy volunteers are envisioned once human MPS-based models and assays translate into valid preclinical platforms. However, industrial adoption of MPS-based assays is progressing slowly due to their complexity. The presentation first highlights rational and state of the art of MPS developments (Marx et al., 2020). Subsequently, examples of human single and multi-organ models, such as human bone marrow (Sieber et al., 2017) and human liver-thyroid co-culture will be introduced. The underlying universal microfluidic HUMIMIC<sup>®</sup> platform of a size of a microscopic slide integrating an on-chip micro-pump and capable to maintain various organ model combinations or single organ equivalents of 16 different human tissues will be described. Challenges of industrial adoption of the platform, with the focus on models supporting repeated dose toxicity testing and simultaneous evaluation of safety and efficacy aspects (Huebner et al., 2018) will be discussed. Furthermore, the creation of more complex physiology-based autologous multi-organ arrangements (Ramme et al., 2019), mimicking adsorption, distribution, metabolism, excretion and crucial organismal feedback loops will be pointed out. We finally, introduce the concept of universal physiological templates (Dehne and Marx, 2020), which might pave the way towards on-chip patient models. Design criteria, aspects of long-term performance, their impact on the drug development paradigm and ethical issues will be elaborated.

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**Presentation:** Oral

948

## A standardized platform for miniaturized cortical organ

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Human Induced Pluripotent Stem Cell (iPSC) derived models of the developing brain are a widely pursued endeavour that has already had some success with regard to psychiatric and neurodevelopmental disorders. The brain organoid (3D) approach has gained increasing traction because of its viability for long-term culture and the superior modeling of developing human brain cytoarchitecture, in particular compared to shorter term monolayer culture. Existing 3D models, however, suffer from considerable variability due to the complex and heterogeneous nature of the free floating structures. Here we describe an iPSC-derived adherent long-term neural culture that models key aspects of neural cortical development. By differentiating neural progenitor cells (NPCs) in the microenvironment of a 384-well plate, it allows for the generation of highly organized neural cultures that resemble early structure formation of the human cortex, with diverse cell types that can be grown and matured for more than 9 months. These miniaturized cortical organoids contain neurons, astrocytes and myelinating oligodendrocytes. There is rudimentary organization of the deep and upper layer neurons, and sufficiently mature to generate dendritic spines. Through cell-type specific transgenic expression of the genetically encoded calcium indicator GCaMP6, the resulting neurons exhibit robust activity *in situ*, for which the overall cortical organoid demonstrates frequent synchronous network bursts. Differentiation of NPCs in the 384-well plate format is highly reproducible and enables the possibility for high throughput screening and mechanistic pathophysiological studies of neuropsychiatric disorders. In summary, we have developed a miniaturized 3D cortical organoid model with a similar level of accessibility to monolayer culture, with the longevity and organizational benefits of organoid (3D) culturing.

**Presentation:** Oral

949

## Towards animal-free *in vitro* neurotoxicity testing and seizure liability assessment

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The demand for drugs targeting the central nervous system (CNS) is rising. Because these drugs have to cross the blood-brain-barrier, they possess a high risk of inducing seizures. Seizures are severe and life threatening adverse side effects. During a seizure, neurons fire in a hyper-synchronous and hyper-active manner. Currently, the seizurogenic potential of a drug is investigated with *in vivo* or *ex vivo* animal experiments. This research is time consuming, expensive and ethically debated. Also, laboratory animals differ from humans and therefore outcomes are not always predictive for human risk. There is thus a clear need for alternative test strategies. The introduction of human induced pluripotent stem cell (hiPSC)-derived neurons offers excellent biomedical research opportunities.

We investigated the suitability of these hiPSC-derived neurons for *in vitro* neurotoxicity testing and seizure liability assessment. Neurons were cultured on micro-electrode arrays (MEAs). This high-throughput state-of-the-art technique allows for non-invasive real-time measurement of neuronal activity and (network) bursting behaviour. Monocultures with solely neurons formed active neuronal networks. Addition of astrocytes made the networks more complex and resulted in (network) bursting and synchronicity. Additional changes in the ratio of excitatory glutamatergic and inhibitory GABAergic neurons further improved the model system and made the network activity better resemble the human *in vivo* situation. MEA recordings showed that *in vitro* exposure of these co-cultures to known seizurogenic compounds results in increased network activity, indicating the seizurogenic potential of the compound.

Our research showed that hiPSC-derived neuronal co-cultures cultured on MEAs can already be used for *in vitro* seizure liability assessment, thereby paving the way towards animal-free neurotoxicity testing. This research led to Dr. Tukker's nomination for the Hugo van Poelgeest Award. The nomination put her research in the spotlight, resulting in (radio) interview requests. It increased her motivation to pursue a career focussing on animal-free models.

**Presentation:** Oral



950

## Björn Ekwall Memorial Foundation 2020 award – “Non-animal methods for toxicity testing – Good In Vitro Method Practices and the pursuit of truly animal free methods”

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Dr. Coecke has a Science, Technology, Engineering and Mathematics-based education (Engineer Biotechnology, Free University Brussels) complemented with a Life Sciences PhD in the faculty of Medicine and Pharmacy. She has been managing and leading scientific teams since the 90's starting in a Belgian Pharmaceutical company and then joining ECVAM at the European Commission Joint Research Centre (EC JRC). Dr. Coecke has more than 30 years' experience in *in vitro* toxicology, including research and activities related to the development and validation of new *in vitro* cell and tissue-based methods and knowledge-based mathematical systems to improve chemical safety assessment. In 1994 she was already awarded the International Price from Foundation for the Substitution of Animal Experimentation in Luxembourg based on her work in the field of metabolism and novel *in vitro* cell and tissue culture systems in general. Last year she received in Varese, Italy, the Women of the year Award demonstrating that by interacting and doing voluntary work closely with European citizens, we can show the added value of our scientific work. A significant part of that scientific work has been dedicated to leading the development and implementation of internationally accepted quality standards, such as Good Laboratory Practices (GLP), for *in vitro* methods. She has been instrumental in the development of the EC JRC's guidance document on Good Cell Culture Practice (GCCP) and the OECD's guidance document on Good In Vitro Method Practices (GIVIMP). Dr. Coecke has established and currently manages the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL), which includes 35 high quality laboratories across Europe, and she is coordinator of a large EU-NETVAL validation study for the detection of chemicals that may disrupt normal thyroid function. She published over 100 *in vitro* toxicological peer reviewed papers, book chapters and has been invited speaker and lecturer in many international conferences and fora.

In her lecture, Dr. Coecke will present insights from her 30 years extensive work related to the development and validation of novel *in vitro* methods, how to promote best practices, and how we can strive to make our non-animal methods truly animal free.

**Presentation:** Oral

1119

## Enabling animal-free safety assessment of cosmetics globally: Introduction

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To achieve animal-free safety assessment of cosmetics globally, it is important to harmonize legislation and regulations to allow flexibility in safety assessment approaches. In addition, it is important to enable local capacity in safety assessment using data generated from non-animal approaches, including *in silico*, *in vitro*, and human-based data. A large collaboration has been established to carry out these functions, including the creation of a program to familiarize global stakeholders with using new types of information and approaches for evaluating safety of cosmetic products and ingredients.

**Presentation:** Oral

1120

## Global cosmetics regulatory landscape

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The regulatory landscape covering cosmetics varies greatly in different countries, from regulatory prohibitions on testing and sales of cosmetics and/or cosmetic ingredients on animals, to countries where animal testing is allowed or even required. In addition, different legal and regulatory structures must be navigated in different countries. Understanding this landscape is critical to understanding the needs and implementing solutions that will support animal-free safety assessment of cosmetics globally.

**Presentation:** Oral



1121

## AFSA cosmetics module on consumer exposure

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Due to the high individual exposure, safety assessment of cosmetics is necessarily exposure-led. Several well-established use and habit factors are considered when measuring or predicting exposure to specific product types and use scenarios. The certainty of the exposure estimate can be increased to match need by using a tiered approach, beginning with simple data and modelling approaches, and progressing to more sophisticated data and complex modelling resulting in increased accuracy and certainty as needed.

**Presentation:** Oral

1122

## AFSA module on internal exposure

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Refinement of exposure estimates can be achieved by measuring and modelling the absorption, distribution, metabolism and excretion (ADME) of a cosmetic ingredient following exposure. Models are built using understanding of biological processes combined with historical data and using data from *in vitro* cell-based assays. In this way, the life cycle of the substance within the individual can be estimated. In addition, using reverse-dosimetry modelling, correlations can be made between *in vitro* cellular and *in vivo* blood concentrations. This allows the establishment of internal exposures related to biological activities measured *in vitro*.

**Presentation:** Oral

1123

## AFSA module on integration of *in vitro* data to establish margin of safety

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*In vitro* methods provide a range of data related to activity related to either specific possible adverse outcomes, or the potential for systemic acute or repeat dose toxicity. These data can be of a wide variety that must be integrated to achieve a numerical result that can be compared to exposure to estimate a safe margin of exposure (MoE).

**Presentation:** Oral

1125

## Advancing read-across practice and applications for the evaluation of data-poor environmental chemicals within the U.S. EPA PPRTV program

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The U.S. Environmental Protection Agency derives Provisional Peer-Reviewed Toxicity Values (PPRTVs) that support the Superfund program in chemical-specific clean-up efforts. However, many chemicals found at contaminated sites lack epidemiological or animal toxicity data necessary for traditional hazard identification and dose-response analysis, preventing regulatory agencies from establishing health reference values that inform clean-up levels. To overcome this challenge, the PPRTV program has developed and implemented a read-across approach for screening-level quantitative assessment of target chemicals with limited *in vivo* toxicity information. The methodology relies on a weight of evidence approach for the identification and evaluation of suitable analogues based on structural, toxicokinetics and toxicodynamic properties. Case study examples will be presented, outlining lessons learned and future outlooks for advancing and expanding read-across applications. Important considerations for problem formulation, target chemical analysis, analogue identification and evaluation and incorporation of data derived from new approach methods (NAM) and tools will also be discussed to increase the transparency and scientific rigor of read-across evaluations. This work emphasizes the integration and translation of computational and expert-driven



techniques in chemical assessment with the potential to inform a broad landscape of regulatory decision-making. The views expressed in this abstract are those of the authors and do not necessarily reflect the views and policies of the U.S. EPA.

**Presentation:** Oral

1132

## SR and meta-analysis of animal studies

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In relation to the reproducibility of animal studies, systematic reviews provide an important diagnostic. That is, they can tell us something about the totality of the evidence; the range of circumstances under which efficacy is or is not seen (Usui et al., 2021); the risks of bias with which the original research was conducted; and the likelihood of a role for publication bias. Armed with a systematic review, research users can reach an informed judgement of whether further research in animals is required (“cis-lation”), or whether research in for instance human subjects is now warranted (“trans-lation”).

Most *in vivo* research does not report simple measures which might reduce the risks of bias, and the fact of their aggregation in a systematic review does not remove this problem. However, describing the provenance on which claims for efficacy are made may help contextualise the claims made in non-systematic literature summaries.

However, a recent study of 442 systematic reviews published from 2015-18 (Hunniford et al., 2021) showed that only 20% mentioned an *a priori* study protocol, and less than half had conducted a risk of bias assessment. This has provided the impetus for the development of a PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) extension for systematic reviews of *in vivo* data, currently in progress.

Finally, concerns about risks of bias in *in vivo* research are not sufficient to drive us to the use of non animal alternatives, until we can show that attention to risks of bias in the *in vitro* literature is not a problem. Preliminary data suggests that reporting of risks of bias in the *in vitro* literature is several fold lower than in the *in vivo* literature.

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**Presentation:** Oral

1180

## Field vs laboratory 3R. It's not about what we do with the animals. It's about the research and its setting!

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As the eldest brother, I learned early that you cannot expect your baby sister to run before she can walk. If team-speed is crucial, you have to support her. In 3R-implementation, wildlife biologists are currently the “baby sisters”. Big Brothers and Sisters in 3R, please help us to learn how to walk and run, and maybe even carry us for a while.

In field ornithology, birds and their biology are generally the object of study *per se*, not a means to study other things. When I put GPS-trackers on wild geese, my aim is *not* to study the effect of the chemicals in the collars, but the movements of the geese. Such basic research is essential for the level of knowledge we have about the multitude of species and their extremely complex environment. Forcing wildlife research to apply hypothesis-driven experiments (the first “commandment” in the PREPARE guidelines) and to put most R&D effort on *Replacement* is counterproductive for the implementation of the 3Rs in this field. A “Wild-goose-on-a-chip” is simply not a viable option, neither for gaining biological insight nor for conservation and management. Not now, probably never.

Field ornithologists are taking their first 3R steps. I have shown how we can learn to avoid redundant measurements to reduce handling times and I routinely use moult feathers instead of blood sampling for population studies. Currently, Johan Lindsjö and I study the various steps in bird ringing on the overall time birds are impeded from their natural routines. Small steps towards improved 3R implementation. Field ornithologists are eager to speed up the process, but please, do not run them down. Help them instead.



1181

## **Evaluating the welfare of wildlife: Identifying priorities**

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Animal welfare has been considered for farmed species for many years, but increasingly we are now considering the impact on welfare of all sentient species. Although wildlife are not owned, and may not be considered in welfare legislation, human interactions can both directly and indirectly impact on the quality of life of wildlife. As part of a study to identify the welfare issues most likely to impact on the welfare of wildlife, we conducted a Delphi study with experts in the field of wildlife and welfare to determine the main issues. Fifteen wildlife experts were recruited and took part in an anonymous discussion board phase for 2 weeks, with the aim to identify the main welfare issues relevant to wildlife in the UK. Using Thematic analysis we identified 54 welfare issues from this process, falling under 4 major themes: 1) Lethal wildlife management and population control; 2) other direct human activities (such as tourism, wildlife capture and handling, trade); 3) impacts on the environment (such as habitat loss, environmental contamination, release of non-native or invasive species) and 4) disease issues. Experts were then asked to evaluate the severity, duration and prevalence of each issue, on the basis of their experience and perception, using a 6-point Likert scale. After 3 rounds of evaluation a final prioritisation list was derived, considering the perceived prevalence of issues (population level) and the impact on the individual (in terms of severity and duration of suffering). The most important issues identified were aspects of lethal wildlife management in terms of the impact on the individual and long-term captivity at the population level. The majority of the issues that were prioritised were those that involved direct human activities, followed by human actions that altered the environment to the detriment of wildlife welfare.



## Poster Presentations

14

### Linking LRI AMBIT chemoinformatic system with the IUCLID6 substance database to support read-across of substance endpoint data and category formation

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Read-across and category formation are indispensable techniques in safety assessments of chemicals. The read-across approach is used on average in 20% of the Endpoint Study Records, while (Q)SAR is used in less than 1% of the dossiers, according to European Chemical Agency reports. Although many tools are available, only a limited number is capable to provide easily accessible data on substance identity, composition together with chemical structures and high-quality endpoint data.

The AMBIT software (<http://cefic-lri.org/toolbox/ambit/>), funded initially within the CEFIC LRI programme, provides a web service and user-friendly web interface to a chemical database, various chemical structure search facilities and toxicity prediction models. The AMBIT data model was further extended to support substances with complex compositions and substances experimental data which allows importing data from the International Uniform Chemical Information Database (IUCLID6) as well as other sources. Currently AMBIT supports manual upload of i6z files exported from IUCLID<sup>6</sup> or semi-automatic import via IUCLID Web services. The chemical structures already contained in AMBIT are automatically linked to constituents/impurities/additives of the imported substances. The flexible data storage and visualization allows for user friendly presentation of study data (physicochemical properties, environmental fate, ecotoxicological and toxicological information) and composition. Comprehensive assessment workflows are developed for read-across and category formation based on all the data available in AMBIT. The assessment workflow facilitates the search for target and source structures, generating data matrices, gap filling and generating assessment reports with predefined formats automatically. The enhanced AMBIT facilitates drafting and improves quality for read-across and category formation and will be a useful tool for experts responsible for substance assessments.

**Presentation:** Poster

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### Protective effect of melatonin on hypoxia-induced cardiomyocyte differentiation of mouse embryonic stem cells

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Hypoxia causes oxidative stress and is known to affect cardiovascular dysfunction and the programming of cardiovascular disease (Giussani et al., 2012). Melatonin is the hormone released primarily from the pineal gland and has been proven to be an antioxidant (Pandi-Perumal et al., 2006). Melatonin promotes the expression of antioxidant enzymes such as superoxide dismutase and catalase (Rodríguez et al., 2004). Differentiation of cardiomyocytes is important because abnormal cardiomyocytes differentiation can lead to cardiovascular dysfunction and cardiovascular disease. To confirm the effect of hypoxia on the differentiation of mouse embryonic stem cells (mESCs) into cardiomyocytes, hypoxia condition induced during the differentiation period. mRNA expressions of cardiac-lineage markers (Brachyury, Tbx20, and Ctn1) decreased at differentiation 2-10 day. The expression of hypoxia marker, Hif-1 $\alpha$ , was increased in the hypoxia condition-plus mESC differentiation 2-10, 6-10, and 2-10 day, but melatonin receptor Mtnr1a mRNA expression was reduced in the hypoxia condition-plus mESC differentiation 6-10 and 2-10 day. To confirm the effect of melatonin against the hypoxia condition, melatonin was treated. Beating ratio and the mRNA expression of cardiac-specific marker (Ctn1) restored in 500  $\mu$ M melatonin-plus hypoxia condition. The level of Hif-1 $\alpha$  protein decreased in melatonin (100, 500  $\mu$ M)-plus hypoxia, but the Mtnr1a mRNA expression was increased. In these conditions, the expressions of p-ERK and Bax proteins decreased, but the levels of p-Akt, PI3k, and Bcl-2 proteins increased. Melatonin has been shown to mitigate hypoxia via the ERK pathway in the differentiation of mESCs into cardiomyocytes. The expression of Mtnr1a mRNA was increased during the differentiation of mouse stem cells into cardiomyocytes, indicating that melatonin may



affect cardiomyocyte differentiation. These results suggest that melatonin may protect against hypoxia in cardiomyocyte differentiation of mouse embryonic stem cells.

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**Presentation:** Poster

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## An *in vivo* screening device platform to reduce animal experiments

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Every year, millions of animals are bred to assess the impact of substances, materials and treatments on human health and the environment. Although a lot of effort is being placed in finding suitable *in vitro* model systems to replace *in vivo* testing, currently developed solutions fail to recapitulate biological complexity. Therefore, *in vivo* experiments are still necessary. Enabling technology allowing reduction of experimental animals would impact tremendously the associated costs and ethical issues. Here, we have explored an alternative route that can potentially abate the number of lives and expenses in animal testing through the development of implantable three-dimensional (3D) screening systems. This new technology platform allows a 9-fold reduction of animals used in preclinical testing. We will present how such devices can be fabricated in multiple formats and with different materials, and their validation for use in cell therapy, biomaterials, and drug screening applications.

**Presentation:** Poster

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## *In vitro* approach for assessing respiratory toxicity in human lung cells

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Approaches to efficiently and effectively assess the toxicity of chemicals on the human respiratory tract using *in vitro* systems would provide useful information to inform product development and risk management decisions. Presented here is an approach to help better understand the appropriate *in vitro* system to use and the biological markers to monitor based on the test chemical under evaluation. In this study, BEAS-2B cells (a human bronchial epithelial cell line) were exposed to various concentrations (0.72 ppm, 25 ppm, and 85 ppm) of triethoxysilane vapor at the air-liquid interface using a capillary dosage unit coupled to a VITROCELL 6/4 exposure module. Triethoxysilane is an industrial chemical classified as a GHS category 2 inhalation toxicant based on rat acute inhalation toxicity testing. A significant concentration-dependent decrease in cell viability (resazurin-based assay) and increase in cytotoxicity (lactate dehydrogenase assay) was observed after exposure to the triethoxysilane (test chemical) and nitrogen dioxide (positive control) as compared to clean air (negative control). A significant increase in expression of inflammatory markers (interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, interferon-gamma (IFN- $\gamma$ ), and tumor necrosis factor-alpha (TNF- $\alpha$ )), determined by Meso Scale Discovery technology, was observed at 25 ppm. Additional work is underway to test other silanes that vary only in their carbon length to determine if this *in vitro* system can detect the decrease in toxicity that correlates with increasing carbon-chain length and to determine the advantages of using a 2D cell line (BEAS-2B cell) versus a 3D human reconstructed tissue model. Overall, these results will evaluate the utility of an *in vitro* system to predict the likelihood of a chemical to cause portal-of-entry effects on the human respiratory tract and could be a useful approach to rank chemical toxicity.

**Presentation:** Poster



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## Successes and challenges in networking and promoting 3Rs within Italian universities. The Centro 3R experience

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The Italian Interuniversity Center for the promotion of 3Rs principles in teaching and research (Centro 3R) was established in early 2018. It currently boasts 7 member universities (University of Pisa, University of Genova, Polytechnic of Milan, Polytechnic of Turin, University of Pavia, University of Milan-Bicocca and the Campus-Biomedico in Rome), over 350 members and brings together a wide range of expertise in disciplines such as pharmacology, engineering, law, biology, medicine and philosophy.

A characteristic of the Centro 3R is its inclusivity and its scientific, rational and evidence-based approach to the question of humane experimentation in all fields of research. In fact, acknowledging that animal research is still necessary in some areas of development (e.g., drugs) and research (e.g., animal science), it seeks to promote responsible research and humane methods as a general approach to their scientific enquiry. Thanks to this inclusive and open approach, it is becoming a point of reference for teaching resources in Italian academia and a platform for discussions.

Many challenges still need to be addressed, particularly the dichotomy in Italian society, which seeps up through the grass roots and permeates the scientific community. This country was in fact the last one to transpose and implement the EU Directive 63/2010 after a heated debate in the Parliament, as well as in the civil society, between supporters and opponents to animal testing (Pavone, 2015). The Centro 3R is creating a common ground for scientists, enabling discussions and networking between individuals, groups and disciplines.

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**Presentation:** Poster

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## Repeating past mistakes: The banality and futility of nowadays cigarette smoke-related animal experimentation

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Since the 1950s experiments have been performed with different animals like mice, dogs and monkeys, forced to inhale, ingest or otherwise receive large amounts of cigarette smoke (CS) or its derivatives. Because of a low reproducibility between different species and a poor transferability to humans, these experiments have been widely criticized by the scientific community and partly used as an argument by the cigarette industry attempting to deny the link between CS and lung cancer. Eventually, despite these experiments, the collective results from multiple large-scale epidemiological studies have conclusively proven that CS is the leading cause of lung cancer and exacerbates many other severe diseases.

Although animal experiments have long been deemed unreliable in assessing the effects of CS in humans, such experiments are still being performed in multiple laboratories. Amongst them are extremely invasive procedures associated with immense animal suffering, including acute trauma, hemorrhagic shock and massive inflammation (Bucher et al., 2017; Jia et al., 2018; Hartmann et al., 2019). Furthermore, with small modifications many studies are merely repeating the effects already described in humans and are frequently failing to reproduce the results of clinical data.

The banality of the outcomes, i.e., that CS contributes to various lung-related health problems, the repetition of studies already performed in patients and the contradiction of clinical results reveals the extreme inadequacy of using animals for the analysis of CS-related diseases in humans. Luckily, many human-oriented, innovative and personalizable methods like precision cut lung slices, 3D lung epithelium models and lung-on-a-chip systems are readily available and approved for regulatory purposes. Here, we show recent animal experiments and human-based research on CS effects. Taken together, we regard the fact that severely harmful animal experiments are still being performed as scientifically and ethically unjustifiable and demand their immediate replacement with more suitable *in vitro* techniques.

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**Presentation:** Poster



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## Systematic reviews to replace animal experiments for optimizing experimental design

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Systematic reviews (SRs) may be used to replace specific animal experiments. In Hannover, we perform systematic reviews of animal studies. Amongst others, we focus on the use of SRs to optimize experimental design of animal studies, by analyzing the effects of changes in the experimental design, e.g., using meta-analyses. This can effectively replace animal experiments. Several SRs analyzing experimental design are in progress or have recently been published. The results will be combined and presented at the conference.

We will first address selection of the optimal animal model, based on three case studies: cystic fibrosis (CF), rheumatoid arthritis (RA) and risk-taking behavior (RTB). For CF we show how a large mapping review of all published animal models with the outcome measurements can aid model selection (Leenaars et al., 2019a). For RA, we analyzed the animal data from a larger review of methotrexate studies to compare the common RA animal models for welfare-related outcome measures (body weight and paw swelling). For RTB, we provide an overview of published models and variations in their experimental design.

We will next show the effect of experimental design factors using meta-regressions. General factors comprise, e.g., sex, time of day and type of control condition, and are shown for corticosterone concentration in mice (Leenaars et al., 2019b). Microdialysis of adenosine will be shown as a case study of a detailed analysis of an experimental technique (van der Mierden et al., 2018).

Based on these five case-studies, we conclude that, when an experimental technique or model has been used repetitively, literature data can be used to predict the effects of experimental design parameters, without performing animal experiments.

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**Presentation:** Poster

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## Systematic reviews to replace specific animal experiments for answering biological questions

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Systematic reviews (SRs) may be used to replace specific animal experiments. In Hannover, we perform systematic reviews of animal studies. Amongst others, we focus on the use of SRs to answer biological questions with increased power compared to individual experiments, effectively replacing larger animal experiments. We will also show how SRs can be used to provide an evidence map, showing where data are available, and where there are remaining gaps. This presentation will use several SRs as an example, focusing on neurochemistry, sleep and circadian rhythms.

Adenosine increases in the basal forebrain during sleep deprivation, while results for other brain regions are as yet still inconclusive. (Leenaars et al., 2018a) Amino acid levels vary with brain region and circadian cycle (Leenaars et al., 2019), and results cannot be generalized over the brain for most amino acids. Sleep deprivation increased glutamate and GABA exclusively in the cortex. Histamine was low during sleep, but high during sleep deprivation and wakefulness, irrespective of brain area. Monoamine levels also vary with brain region and circadian cycle (Menon et al., 2019). Noradrenaline and serotonin levels decrease from wakefulness to slow wave sleep and decrease further during Rapid Eye Movement sleep. In contrast, monoamine levels generally increased during sleep deprivation, and sometimes remained high even during subsequent recovery.

A large mapping review retrieved 2331 papers describing intracerebral amino acid measurements. (Leenaars et al., 2018b). The number of references by compound varies from only 6 references for proline to 1876 references for glutamate, showing that the relative evidence-density for these compounds varies.

Based on these case-studies, we conclude that, when multiple studies address roughly the same research question, literature data can be used to answer this question with increased power, without performing additional animal experiments. The use of SRs to answer biological questions is however limited to questions for which sufficient primary data are available.

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**Presentation:** Poster

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## Promoting the replacement of the 3Rs principle: Short courses

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When Russell and Burch postulated the 3Rs principle, the R of replacement was considered the first option and when this was not possible then refinement and reduction should be considered (Russell and Burch, 1959). A recent paper presents the results of a survey of participants on mandatory courses in laboratory animal science in different European countries with the objective to know among others the perception of researchers on the need of animal experimentation and the 3Rs principle. Surprising the general answer was that refinement is best and replacement is only supplementary to the animal use, opposite to the postulate of Russell and Burch (Franco et al., 2018). There is need for specific training on 3Rs and especially in the replacement (Daneshian et al., 2011).

With the objective of improving the knowledge of the researchers on replacement we have organized short courses for continuous learning of people working with laboratory animals. The main objectives of these courses were to present the more relevant methodologies developed to replace animals and the best way to do the search of alternatives. For these reasons, experts on the different methods presented them, with especial attention to the advantages versus animal models. Among these methodologies there are organ-on-a-chip, 3D models, computational models, etc. The courses were scheduled on two afternoons with four sessions each day in order to allow the attendance and compatibility with work.

The attendants expressed their interest and the lack of knowledge on the alternatives proposed. This kind of initiative promotes the replacement and tries to change the mind of the scientists towards a non-animal research.

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**Presentation:** Poster

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## cellasys #8: A microphysiometric assay to assess enhanced cell culture media

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A new protocol based on a microphysiometric system (McConnell et al., 1992; Hartung et al., 2010; Wiest et al., 2016; Alexander et al., 2018; Brischwein and Wiest, 2019) to analyze cell culture medium (CCM) is described. With the presented cellasys #8 protocol, significant data can be gained in 24 h compared to conventional weaning experiments which need several weeks to perform. First, L929 cells are supplied for 6 hours with DMEM + FBS reference medium to gain initial data. In a second step, 6 hours of the investigated test medium is supplied to see if there are any changes in cellular vitality or morphology. Then again 4 hours DMEM + FBS and 4 hours of test medium to monitor if the effect of the CCM to the cells is reversible. The experiment ends with 4 hours of test medium + 0.2% SDS to induce cell death. In the presented work, two chemically defined CCM and a common serum containing medium DMEM + FBS were tested on the L929 cell line. Compared to the reference medium, cells in the DMEM/Ham's F12 + ITS medium pursue a loss in adherence, but no decrease in extracellular acidification rate. This was substantiated by the observation that the acidification rate remained constant, and the impedance recovered after changing back to the reference medium. Cells in NCTC 135 retained their impedance values but lost vitality. It seems reasonable to suppose that cells in NCTC 135 medium are slowly suffering as indicated by a slow decrease in impedance and highly fluctuating acidification rates. Further experiments for the presented method could be the improvement of the DMEM/Ham's F12 + ITS medium. With the new method electrical cell-substrate impedance and extracellular acidification responds of the



cells can be measured immediately and consequently the quality of new CCM can be quantified.

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**Presentation:** Poster

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## Insights on P-glycoprotein ligand interactions from molecular dynamics simulations

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P-glycoprotein (P-gp) is a transmembrane protein belonging to the ATP binding cassette superfamily of transporters; it is a xenobiotic efflux pump that limits intracellular drug accumulation by pumping the compounds out of cells. P-gp contributes to a decrease in toxicity and possesses broad substrate specificity. It is involved in the failure of many cancer and antiviral chemotherapies due to the multidrug resistance (MDR) phenomenon in which the membrane transporter removes the chemotherapeutics from the targeted cells. Understanding the details of the ligand-P-gp interaction is, therefore, crucial for the development of drugs that might overcome the MRD phenomenon and for obtaining a more effective prediction of toxicity. In this work, a series of molecular dynamics simulations of human P-gp (hP-gp) are developed using the hP-gp 3D structure. The molecular dynamics simulations are performed on a set of

nine compounds, including some well-known ligands of P-gp and some non-active compounds. The 3D structure of hP-gp in the inward-facing conformation is embedded into a POPC lipid bilayer and 500 ns molecular dynamics simulations are performed. We aim to analyze the behavior of several molecules inside the binding pocket, assess if there is a significant difference between the molecular interactions of substrates and inhibitors, to identify any motion patterns and evaluate the stability of the binding interactions of the compounds during the transport.

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## USG & RJ Lee *in-vitro* study on the biopersistence of respirable mineral fibers with existing and refined test methods

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Animal testing has been used to evaluate the biopersistence of respirable mineral fiber materials for decades. There are many ethical, economic and logistical issues associated with conducting animal studies, and alternative test methods are needed. The purpose of this study was to refine existing test guidelines for *in-vitro* acellular dissolution testing and to correlate the results with a previous animal study of the same mineral fiber composition.

An *in-vitro* test was performed on the respirable portion of a specific mineral wool composition (referred to as Fiber 22) per guidelines established by the European Insulation Manufacturers Association (EURIMA). Fiber dissolution characteristics were evaluated over a 28-day period within a flow-through test cell using a modified low calcium Gamble's solution maintained at a pH of 4.5 and a temperature of 37°C. These conditions simulated the environment within phagolysosomes of alveolar macrophages of the lung. Fiber dissolution rate constants (Kdis) were determined on the basis of dissolution of five elements (Al, Ca, Fe, Mg, and Si) and of gravimetric analysis. Higher Kdis values correspond to increased biosolubility of the fiber and *vice versa*.

For the *in-vitro* biopersistence test, elemental Kdis average values ranged from 142 for Si to 636 ng cm<sup>-2</sup> h<sup>-1</sup> for Mg. The average Kdis for the summed mass loss of all five elements was 358 ng cm<sup>-2</sup> h<sup>-1</sup>; the gravimetrically determined Kdis was 453 ng cm<sup>-2</sup> h<sup>-1</sup> for the fiber overall.

The results of the *in-vitro* biopersistence test for the Fiber 22 composition provided information that can be used as a basis for comparison to favorable results observed in animal studies. It suggests that *in vitro* fiber dissolution testing can be used to



predict the carcinogenic potential of a particular fiber's composition in place of, or to greatly reduce the need for, animal testing.

**Presentation:** Poster

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## Monitoring innovation and societal impact of biomedical research

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Chronic diseases, such as Alzheimer's disease, cancer, and cardiovascular disease are becoming increasingly prevalent in Western countries. Such noncommunicable diseases account for 71% of all deaths globally and are generally the result of a combination of genetic, physiological, environmental, and lifestyle factors (WHO, 2018). Over the last two decades, the European Commission (EC) has invested extensively in biomedical research to increase understanding of the mechanisms underlying disease pathogenesis and consolidation. However, despite this research and economical endeavor, the clinical failure rate in drug development remains very high, with an overall likelihood of approval from Phase I around 9.6% and almost a 95% failure rate in drugs entering human trials (Seyhan, 2019). It is increasingly recognized that advanced human biology-based models contribute to a deeper understanding of human health, how diseases emerge, develop, and spread, and drive the development of safe and effective therapeutics. There has been a noticeable paradigm shift away from the reliance on whole animal models to study human physiology, pathology, and pharmacology toward the utilization of human-relevant model systems. To increase the understanding of the scientific and societal impacts of animal and non-animal approaches in biomedical research we have developed suitable indicators to retrospectively assess the impact of EC-funded research activities in the fields of Alzheimer's disease, breast cancer, and prostate cancer. Through the collection and analysis of quantitative and qualitative research, we aim to understand how EC-funded research has contributed to innovation and scientific breakthroughs, how scientific results have translated into socioeconomic impacts of benefit to society, the scientific methods and research approaches contributing to the advances made, and measure return on investment in biomedical research.

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**Presentation:** Poster

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## Workshop on the implementation of next generation risk assessment (NGRA) for systemic toxicity

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Next Generation Risk Assessment (NGRA) is an exposure-led, hypothesis-driven approach integrating new approach methodologies (NAMs) to ensure the safety of consumer products without the use of animal data. We recently applied an ab initio tiered framework (Berggren et al., 2017), based upon the ICCR principles (Dent et al., 2018), to a hypothetical safety assessment of coumarin in a shampoo, face cream and body lotion. This provided practical experience in applying NAMs as part of an NGRA framework. A workshop was held in October 2019, which focused on the approach to systemic toxicity risk assessment demonstrated in this case study and the underpinning mechanistic science.

Key areas discussed include:

1. Communication and framework development – There is a need for a more general and universal framework for approaching an ab initio NGRA. Also, communication of the inherent uncertainties and the distributions generated for points of departure (PoD) and margins of safety are essential.
2. Decision making – Utility to make informed, meaningful de-



cisions on the available data is a key part of safety assessment, but knowing when we have enough data to be confident can be challenging.

3. Optimal assay design – Characterizing and standardizing the design of the assays used can reduce the uncertainty of assay outputs and thus of the PoD for internal and external exposures. This topic was discussed in the context of incorporating metabolism and clearance.
4. Making the most of benchmarking – The use of suitable benchmarking reference chemicals is important for both assay evaluation and for interpreting the risk associated with margins of safety derived from NAM-based PoDs.

This example illustrates how case studies are an impactful method for communicating the current capabilities of NGRA, ultimately driving conversations that will lead to change in the understanding and acceptance of non-animal approaches to safety assessment globally.

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**Presentation:** Poster

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## R-ODAF: Omics data analysis framework for regulatory application

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While the use of omics techniques is increasing in research, their application in regulatory toxicology is still extremely limited. Omics are commonly criticized for not being sufficiently reliable for regulatory application because the output depend on the applied analysis pipeline. This reticence to trust omics data is further magnified by the lack of internationally agreed upon guidelines and protocols for both the generation and processing of omics data. One way forward would be to reach a consensus on the omics data analysis framework for regulatory application (R-ODAF) based on rigorous data analysis. The authors of this study are proposing an R-ODAF for transcriptomics data for three platforms: microarrays, RNA-Sequencing and TempO-Seq. The R-ODAF will then be reviewed and evaluated by the main

regulatory agencies and consensus forums such as the Organization for Economic Co-operation and Development (OECD). This work is running in parallel alongside an OECD initiative to develop a guidance document called a transcriptomics reporting framework (TRF) that will enhance the quality of reporting of omics data when generated for regulatory purposes. The presentation of the project is now published (Verheijen et al., 2020), and the final proposed pipeline will be presented in the event.

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**Presentation:** Poster

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## Corneal edema simulation and therapy in the ex vivo eye irritation test (EVEIT)

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**Introduction:** Degenerative corneal disease often leads to corneal edema and visual loss in humans. One therapeutical approach is the application of deswelling eye drops. Up to now there is 5% saline solution as a typical deswelling agent. These drops initially tend to have a good effect but, over the course of further treatment are prone to rebound and worsen the problem. We wanted to improve those drops and developed the corneal edema test in the EVEIT to avoid animal experimentation.

**Methods:** We modified our standard approach to cultivate rabbit corneas from abattoir in our EVEIT system. For this, we used a culturing medium (MEM) diluted with hypo-osmolal saline solution. Through this, the EVEIT cornea shows considerable edema with thickness of original 500 µm up to 800-1000 µm. By applying various hyper-osmolal solutions we simulate the therapeutical effect of edema reduction.

We used different commercially available preserved and unpreserved hyper-osmolal saline solutions 5% with/without different additives. The goal was to develop a novel formulation more effective than the commercial references, which was expected to improve the long-term stability of corneal deswelling without rebound and without disturbing the corneal epithelial surface.

**Results:** The comparison of 5% saline solution with preserved 5% saline solution and another, unpreserved 5% saline containing hyaluronic acid showed deswelling effects but dependent on preservatives surface damage and rebound swelling. We devel-



oped formulations with comparable effectiveness, lower saline content preventing the rebound effect.

**Conclusion:** There is evidence that the biological system of the EVEIT can not only be employed to identify corrosive substances but is also able to guide clinically the development of new therapeutical substances. Here, we simulate corneal edema, its therapy and reversibility. The replacement of live animal experimentation is realized here with the simulation of high exposure rates and existing therapy regiments close to clinical reality.

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## The ex vivo eye irritation test (EVEIT) system in the distinction between slight and severe corrosives

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**Introduction:** In 2012 we published data on the EVEIT using 38 corrosives (Spöler et al., 2015). The substance set used contained some of the “should not be used” substances according to the analysis of Barroso et al. (2017) in the DRD Database. The authors recommended against involving these substances in test development and/or validation. We re-evaluated the data and focused on the ability of the EVEIT test to distinguish between Cat 1 and Cat 2 or NC corrosives.

**Methods:** All substances used in our tests were analyzed on “should not be used” and whether or not the cornea was the driving factor. We excluded substances that should not be used and those where the cornea was not the driving factor for the resulting categorization.

**Results:** In this analysis we found a 97% specificity and a 98% sensitivity of the EVEIT test to identify Cat 1 substances. One substance with a false positive Cat 2 result in our system was Trichloroacetic acid (TCA) which is GHS-classified as NC.

**Conclusion:** The false positive result is a consequence of superficial burns being the main effect of TCA. In living animals this is subject to shedding of epithelial cells motivated by lid action which is not realized in the EVEIT model system. We introduced a rinsing procedure to simulate epithelial renewal. We are certain that this type of superficial burns, which are healing over the course of an animal experiment, can be simulated in the EVEIT system. Overall, the CAT 1 – detection rate of our model is the highest amongst all live-animal-free experimental approaches used in toxicology.

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**Presentation:** Poster

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## A precisely adjustable, live animal free ocular corrosion model

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**Introduction:** The need for live animal free toxicological tests has increased in numerous branches of science, medicine and industry. In this project we improved the EVEIT *ex-vivo* ocular chemical injury model. Now we can induce and measure ocular corrosive effects on a precisely adjustable and consistent scale.

**Methods:** The model is utilizing the *ex-vivo* organ culture technique employed by the Ex Vivo Eye Irritation Test (EVEIT). Briefly, rabbit corneas from food industry are installed into specialized culturing chambers. Corneas are supplied with nutrients from the endothelial side in a continuous flow. Physiological pressure and flow conditions are realized by an artificial anterior chamber.

A three-axis corrosive applicator workstation (BioFluidix GmbH, Germany) with a specialized impulse-based application mechanism is able to precisely place substance droplets in the nanoliter range contact free onto the corneal surface. Timing and frequency of droplet application can be exactly adjusted, so that multiple positions on one cornea can be treated sequentially.

The depth of corneal injury induced by the substance application is detected by means of a high-resolution OCT system. The quantification of the damage zone in OCT images is performed by analysis software.

**Results:** An application of 10-80 nL of NaOH in increasing concentrations (250 mM, 500 mM, 1000 mM, 2000 mM) to the corneal surface yielded significantly different OCT signals for each concentration used. The detected penetrative effect ranged from superficial to extensive. Further, a direct strong correlation was established between the volume and the extent of the measured OCT signal.

**Conclusion:** With this novel live-animal-free method, we were able to induce ocular corrosive effects in a precise and consistent



manner. The effects, which ranged from superficial to extensive, were controllably and consistently triggered. Thus, this method could provide a basis for future *ex-vivo* investigations into the treatment of ocular corrosive injuries.

**Presentation:** Poster

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## The validation of a semi-high throughput automated ocular toxicity test

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**Introduction:** The necessity of a live-animal-free ocular toxicity test is strongly represented in multiple branches of science, medicine and industry. The Ex Vivo Eye Irritation Test (EVEIT) is providing the platform for various *ex-vivo* testing applications. To improve the efficiency of the test, we adapted an automated substance application apparatus that is able to precisely place quantified test substance droplets in the nanoliter range onto the corneal surface. With this technology we are able to test multiple test substances on a single cornea.

**Methods:** The model is utilizing the *ex-vivo* organ culture technique employed by the Ex Vivo Eye Irritation Test (EVEIT). Briefly, rabbit corneas from food industry are installed into specialized culturing chambers. Corneas are supplied with nutrients from the endothelial side in a continuous flow. Physiological pressure and flow conditions are being realized by the artificial anterior chamber.

A substance applicator workstation with a specialized impulse-based application mechanism is able to precisely place substance droplets contact free onto the corneal surface. Timing and frequency of droplet application can be exactly adjusted, so that multiple positions on one cornea can be treated sequentially.

Categorization into NC, CAT2 or CAT1 is based on observations via OCT and live fluorescein staining immediately after substance exposure and two days later to integrate acute and long-term effects respectively.

**Results:** The application mechanism, in combination with the *ex-vivo* organ culture model, is very precise with a high degree of repeatability. Further, the setup allows for the instillation of minute adjustments. Correlations of application and damage patterns with strong corrosives are highly repeatable.

**Conclusion:** We aim to be able to recreate the results of the substance categorization created in the pre-validation project. The novel application mechanism exhibits a high degree of flexibility which enables us to fine-tune the testing system according to our needs.

**Presentation:** Poster

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## Rethinking carcinogenicity assessment for agrochemicals

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For the past 40 years, questions have been raised about the relevance and regulatory utility of rodent cancer bioassays in human health risk assessment. As a result, a working group of experts from different sectors have formed the Rethinking Carcinogenicity Assessment for Agrochemicals Project (ReCAAP) to determine the appropriateness of and criteria for waiving rodent cancer bioassays for the registration of food-use pesticides. A weight of evidence (WoE) reporting framework, which outlines a suggested assessment of publicly available information, was used to draft carcinogenicity study waivers to determine if sufficient information was available to perform a health protective chronic risk assessment without conducting rodent cancer bioassays. Information used in the WoE include exposure, mode-of-action, physiochemical properties, metabolism, and sub-chronic toxicological data from standard risk assessment endpoints. Using this framework, ReCAAP evaluated 15 pesticides registered over the past ten years with the chemical distribution spanning nine tumor types, 15 chemical classes, and six cancer classifications (including subclasses). The reporting framework criteria and example carcinogenicity waivers will be presented. This effort has established criteria for when the mouse and/or rat cancer bioassay can be waived while ensuring that pesticide human health risk assessments are protective.

**Presentation:** Poster

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## Differential influence of *Streptococcus mitis* on host response to metals in reconstructed human skin and oral mucosa

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**Background:** Skin and oral mucosa are continuously exposed to potential sensitizers whilst hosting abundant microbes which may influence the host response to sensitizers. This host response may also be influenced by the route of exposure, e.g., skin or oral mucosa, due to their different immune properties.

**Objective:** To determine how commensal bacteria, *Streptococcus mitis*, influences the host response to nickel sulfate (sensitizer) and titanium(IV)-bis(ammonium-lactato)dihydroxide (questionable sensitizer) in reconstructed human skin (RHS) and gingiva (RHG).

**Methods:** RHS and RHG were exposed to nickel or titanium, in the presence or absence of *S. mitis* for 24 hours. Histology, cytokine secretion and TLR expression was assessed.

**Results:** *S. mitis* increased IL-6, CXCL8, CCL2 and CCL20 secretion in RHS but not in RHG; co-application with nickel further increased cytokine secretion. In contrast, titanium suppressed *S. mitis*-induced cytokine secretion in RHS and had no influence on RHG. *S. mitis* and metals differentially regulated TLR1 and 4 in RHS, and predominantly TLR4 in RHG.

**Conclusion:** Co-exposure of *S. mitis* and nickel resulted in a more potent innate immune response in RHS than in RHG in line with clinical observations that skin has immunogenic and oral mucosa has tolerogenic properties. In contrast, titanium remained inert even when co-applied with bacteria further supporting that this metal is a non-sensitizer. These results indicate the important influence of commensal microbes and the route of exposure on the host's response to metals.

**Presentation:** Poster

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## Biological data of nonhuman primate during the quarantine periods

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Quarantine is the most important part of research using primates. Unlike other animals, primates are often imported from abroad, which is likely to cause various problems depending on long-distance transportation and changes in the environment. Therefore, it is necessary to establish the proper quarantine methods and check the animal's health condition, especially since each animal's sensitivity to the disease varies depending on the area it was raised in. This study seeks to share the results of general symptoms, weight changes, feed intake, urinalysis, hematological examinations, and clinical cases and treatments that may occur during the quarantine period in 252 cynomolgus monkeys. I believe the above results will help with the management and treatment of animals during quarantine at places where primate experiments are conducted.

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**Presentation:** Poster



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## Early-career researchers advancing 21<sup>st</sup> century science: An educational initiative for human-relevant biomedical research

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Increasingly, scientists, regulators, and the public are acknowledging the need to overcome a cultural and historical reliance on using animals in research. Habitually, animals are used in biomedical research to recapitulate characteristics of human disease and to drive the development of therapeutics. However, human biological, pathological, and pharmacological events often cannot be simulated in animals due to inherent genetic, molecular, anatomical, and physiological differences (Sousa et al., 2017). The advancement of *in vitro* and *in silico* models has led to fundamental improvements in human disease modeling and drug development (McAleer et al., 2019). However, minimal funding and opportunities exist for using human-based models for biomedical research. Understanding the importance of early-stage scientific mentorship, the Physicians Committee designed the Early-Career Researchers Advancing 21<sup>st</sup> Century Science (ERA21) program, to strategically intervene with up-and-coming scientists to help build their careers without using animals, from the start. ERA21 aims to provide education and early-career experiences that will instill practices to shape professional lives for decades. Using a multifaceted approach specifically tailored for emerging biomedical researchers, the ERA21 initiative aims to: increase the understanding of the scientific benefits of using human-relevant research practices, educate emerging scientists about the wide range of modern, human-relevant methods available, and connect them with opportunities in this field. ERA21 offers programs to reach students and early-career scientists and launch promising professions. Activities include educational seminar series, hands-on training and in-depth interactive learning, incentivizing student researchers toward human-relevant projects through travel awards, identifying and connecting students with laboratories using nonanimal methods, a monthly newsletter, social media groups to facilitate networking and share funding and job opportunities, presentations and outreach at relevant biomedical conferences, and more.

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**Presentation:** Poster

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## Customizing animal welfare legislation for animals used in xenotransplantation trials and production in the United States

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Recent advances in overcoming both immunological and pathological barriers across species (Liu et al., 2017) make xenotransplantation a potential solution to ongoing shortage of human organs. Genetically engineered pigs are currently being raised in the U.S. for clinical xenotransplantation trials in humans (Mullin, 2019). The Animal Welfare Act (AWA), enforced by the United States Department of Agriculture (USDA), is the only federal law regulating the treatment of animals used in research in the U.S. (USDA, 2017), while the Center for Biologics Evaluation and Research (CBER), under the Food and Drug Administration (FDA), has regulatory oversight of xenogenic products and xenotransplantation in humans (HSS, 2016). Animals involved in xenotransplantation are not specifically described under the AWA but are addressed under the FDA's "Guidance for Industry" documents instead. These guidance documents do not establish legally enforceable responsibilities and focus primarily on public health concerns rather than the welfare of source animals. Current animal protection laws other than the AWA, such as anti-animal cruelty laws, vary from state to state, while animal welfare monitoring for livestock production relies heavily on voluntary third-party audits and certification programs. Given the unique functions and needs of animals raised for tissue and organ harvest for human use, the production and housing of such animals will necessitate specialized regulatory oversight different than that for animals in food production and non-xenotransplantation laboratory research. Collaboration between the USDA and the FDA is critical as well as mandatory auditing or accreditation for all facilities housing animals for xenotransplantation programs. There is a pressing need for U.S. regulatory authorities to review and revise current federal and state laws in order to customize legislation to protect the welfare of this unique group of xenotransplantation-bound laboratory animals until alternatives and replacement for animal use become available.

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**Presentation:** Poster

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## Three-stage approach for evaluation of a chemically defined cell culture medium for the Caco-2 cell line: Short-term effects, differentiation potential and long-term cultivation

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Finding a chemically defined cell culture medium as a replacement for FBS is a time-consuming and costly process (van der Valk et al., 2018). In this work, we present a three-stage approach for evaluating the chemically defined DMEM/F12+ITS medium (SOP-G200-005\_DME\_F12+ITS) for the Caco-2 cell line. The three stages are increasingly lengthy and identify adhesion, proliferation and metabolism changes of the cell culture. The first stage utilizes the microphysiometric system intelligent mobile lab for in-vitro diagnostic (IMOLA-IVD) to reveal any short-term effects caused by the chemically defined medium (Weiss et al., 2013; Brischwein and Wiest, 2019). The IMOLA-IVD device enables an automated cell analysis by label-free measurements of the acidification rate and impedance. Additionally, the device provides fresh nutrients to the Caco-2 cells regularly by a pre-programmable protocol. Herein, the used protocol cellasys #8 is defined as follows: 6 h DMEM + 5% FBS, 6 h DMEM/F12+ITS, 4 h DMEM + 5% FBS, 4 h DMEM/F12+ITS, 4 h positive control with 2% Sodium dodecyl sulphate (SDS). In the experimental results no short-term effects and cellular stress responses are visible. This suggests that all major nutrients are present in the chemically defined medium. The second stage is a differentiation cultivation in a T25 flask for 40 days while the third stage is a long-term cultivation for 100 days. The qualitative results of these stages obtained by a

light microscope show that the proliferation and adhesion is reduced compared to DMEM + 5% FBS cultivated cells but is constant during the long-term cultivation suggesting that there are no missing nutrients. With the IMOLA-IVD system, the screening time for finding minimal, chemically defined media can be reduced. The real-time measurement of the device shows any cell stress caused by missing nutrients in a chemically defined medium formulation. This enables a rapid first stage screening with a duration of 24 hours.

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**Presentation:** Poster

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## Evaluation of a novel oral mucosa in vitro implantation model for analysis of molecular interactions with dental abutment surfaces

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**Background:** Abutment surfaces are being designed to promote gingival soft tissue attachment and integration. This forms a seal around prosthetics and consequently ensures long-term implant survival. New scalable and reproducible models are necessary to evaluate and quantify the performance of these surfaces.

**Purpose:** To evaluate a novel implantation model by histomorphometric and immunohistochemical characterization of the in-



teractions between human oral gingival tissue and titanium abutments with either novel anodized or conventional machined surface.

**Materials and Methods:** Abutments were inserted into an organotypic reconstructed human gingiva (RHG) model consisting of differentiated gingiva epithelium cells on a fibroblast populated lamina propria hydrogel following a tissue punch. Epithelial attachment, down-growth along the abutment surface, and phenotype were assessed via histomorphology, scanning electron microscopy, and immunohistochemistry 10 days after implantation.

**Results:** The down-growing epithelium transitioned from a gingiva margin to a sulcular and junctional epithelium. The sulcus depth and junctional epithelial length were similar to previously reported pre-clinical and clinical lengths. A collagen IV/laminin 5 basement membrane formed between the epithelium and the underlying connective tissue. The RHG expanded in thickness approximately two-fold at the abutment surface. The model allowed the evaluation of protein expression of adhering soft tissue cells for both tested abutments.

**Conclusions:** The RHG model is the first *in vitro* 3D model to enable the assessment of not only human epithelial tissue attachment to dental abutments but also the expression of protein markers involved in soft tissue attachment and integration. The two abutments showed no noticeable difference in epithelial attachment.

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**Presentation:** Poster

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## Novel home cage activity metrics for postoperative care refinement in a mouse surgical model

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Postoperative monitoring is essential for animal welfare and scientific reliability, as unexpected complication can result in unalleviated pain and impact the quality of data generated. Due to the inter-individual variability between operators when assessing animal condition, objective quantifiable parameters, such as body weight, are often used. These methods can be time consuming and stressful for the animal. In this study we used digital ventilated cages to non-invasively monitor home cage activity in a mouse surgical model. The primary objective was

to identify and validate a novel measure of activity reflective of animal condition during the pre- and post-operative phases. C57BL/6Cr1 male mice were housed three per cage and divided in control, sham and surgery groups (n = 5 cages per group). The surgery group underwent full tenotomy of the right hind limb while sham group underwent skin incision without tenotomy. All animals were weighed weekly and clinically scored three times per week. Operated animals were treated with buprenorphine for three days. While clinical scoring and body weight did not differentiate between groups, post-surgery activity was reduced in both sham (-25%) and surgery (-50%) groups the first night without analgesic injection. From this time point, activity and response to lights-on increased in both sham (p = 0.001) and surgery (p = 0.003 - 0.04) groups. Where the sham group returned to pre-surgery activity levels eight days post-operation, the surgery group activity was less than sham (p < 0.0001) for six weeks post-operation. Analysis of distribution of activity showed similar post-surgery activity reduction in both groups in the front row. Only sham group activity in the front row returned to pre-surgery levels eight days post-operation. Our results show how home cage activity metrics can be used as valuable complementary tool for postoperative care refinement and for sensitive assessments of degree and time to return of function in surgical mouse models.

**Presentation:** Poster

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## The role of the animal welfare body in developing a functional and efficient culture of care

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Each user-establishment has its own and unique Culture of Care which requires a tailor-made attention to address the relevant challenges and issues. Approaches on how to work with Culture of Care has recently been published by (Robinson, 2019) and (Hawkins, 2019). Culture of Care can be a powerful and efficient tool to ensure, promote and advance animal welfare and the practical application of the 3 Rs – or it can be a meaningless phrase with no impact on these matters. This presentation will go through three relevant elements of making Culture of Care functional and effective and how the Animal Welfare Body (AWB) can work with this, making Culture of Care a strong enabler of optimizing animal welfare and the 3Rs.

The three elements are the culture, the desired outcomes and the structures that support and connect these two elements. The Culture of Care can be assessed in terms of “what does it look like”. The outcomes – in terms of “what does it achieve” – can be measured by using Key Performance Indicators (KPIs) to as-



sess its functionality and efficiency. The supporting structures are the different tools that the AWB identify and deploy to transform culture into achievements. These tools are unique for each individual user-establishment. The role of the AWB is to identify and understand the current culture, define the desired animal welfare outcomes and decide which tools are needed to connect these two in order to achieve the outcomes.

This presentation will demonstrate practical examples from each of the three elements and how the Animal Welfare Body can work with them. The examples will have a universal nature and they can in principle be deployed at any user-establishment.

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**Presentation:** Poster

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## Reduction of controls in preclinical clamp studies using a non-linear mixed-effects model

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Historical glucose clamp studies in rat from 2000 to 2015, in total 59 studies with 2567 rats, following almost the same protocol, all with the same control treatment (human insulin) were analyzed with a non-linear mixed effects model (basically a four-parameter logistic model). The purpose was to quantify the variation in the control rats, within and between studies, and quantify how this variation translated into the precision of the estimated relative potency parameter of novel insulin analogues. Simulations based on the fitted model were used to study the value of historical controls in future potency studies. The simulations showed that inclusion of historical information could replace 50% of the control rats.

**Presentation:** Poster

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## Chemically defined formation of spheroids loaded with superparamagnetic iron oxide nanoparticles

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To increase physiological relevance and to develop new therapies 3D spheroids are of increasing interest. The addition of superparamagnetic iron oxide nanoparticles (SPIONs) into the spheroid allows for investigation of new therapeutic strategies (Zhang et al., 2014) without the use of animals (Lei and Schaffer, 2013; Whatley et al., 2014; Marx et al., 2016). L929 fibroblasts were maintained in 25 mL Greiner Bio-One AdvancedTC<sup>®</sup> cell culture flasks in chemically defined DME/F12+ITS cell culture medium. Cells received fresh medium twice a week and were passaged at about 80% confluency once a week. Spheroids were created similar to a previously developed method (Alexander et al., 2018). The 20 nm SPIONs beads were obtained from micromod Partikeltechnologie GmbH (Rostock, Germany) and a stock solution with 100 µg/mL was prepared. Spheroids were prepared in a 96 well-plate with cell-repellent surface (Greiner Bio-One GmbH, #650970). Each well was filled with 190 µL DME/F12+ITS containing 10,000 L929. For the SPION loaded spheroids (SPION-LS), 10 µL of magnetic beads stock solution was added, whereas for the non-magnetic control spheroids (NM-CS) 10 µL of cell culture medium was added. Then the 96well plate was centrifuged at 1000 g for 5 min and finally incubated at 37°C with 5% CO<sub>2</sub>. 100 µL of medium in each well was replaced by fresh DME/F12+ITS (preheated to 37°C) daily. Spheroids were used for the experiments on day 5. For manipulation of the spheroids a neodym magnet (length 25 mm, diameter 25 mm) was used in a distance of approximately 30 mm. The calculated field force acting on the spheroids was approximately 30 mT. To investigate if the SPIONs are incorporated into the spheroids one SPION-LC was transferred to a well with one NMCS. The movement of the SPION-LC due to the applied magnetic field can be seen at <https://youtu.be/4S-oTBloGss>.

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## Modernizing biomedical research and regulatory policies to advance human health

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An economy of human-relevant research methods has developed, and it is rapidly outperforming animal experiments, holding tremendous promise to revolutionize regulatory testing, biomedical research and usher in the age of personalized medicine. The transition away from research relying on animals to model human disease or to predict human responses to drugs or other substances and towards human biology-based methods is changing policy and practice around the globe. Systematic reviews and detailed analyses published in peer-reviewed journals document limitations in translating results from animal experiments to humans for numerous disease areas. For example, the U.S. National Institutes of Health reports a 95 percent failure rate of clinical trials for new pharmaceuticals following preclinical success in animals (NCATS, 2018) because the drugs are either not safe or not effective (U.S. FDA, 2004). A 2015 analysis concluded that the prevalence of irreproducible preclinical research was between 50 and 89 percent, which, at the most conservative estimate, results in approximately US\$28 billion (€25 billion, or £22 billion) per year spent on research that is misleading (Freedman et al., 2015). Governments that mandate a move away from animal experimentation and toward more advanced scientific methods have the opportunity to expand job growth rapidly in science and technology, to reduce healthcare costs for their citizens and to streamline the drug development and toxicity testing process. Consequently, PETA US and its international affiliates have published strategic plans for the US, European, and Indian audiences, the Research Modernization Deal, which offer a robust blueprint on how limitations in animal use, along with the increasing availability of human-relevant biotechnology, can be translated into actions aimed at eliminating inefficiencies (PETA US,

2020). We highlight a number of strategic priorities regarding areas of both regulatory and non-regulatory research where opportunities lie for the immediate and forthcoming replacement of animal use.

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## Certain harms and uncertain benefits in animal models for the study of human depression and anxiety

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Anxiety and depressive disorders, often comorbid conditions, are the most common mental disorders, affecting 4.4 and 3.6 percent of the global population, respectively (World Health Organization, 2017). In efforts to use mice and rats as models to study these illnesses, experimenters employ a variety of behavioral tests, many of which have come under scrutiny due to their over-simplicity, unreliability, and lack of specificity. We explore various aspects of validity (construct, face, translational, internal/external) of the most commonly used neurobehavioral assays in animal models of depression and anxiety, including the forced swim test, tail suspension test, elevated plus maze, zero maze, light/dark box test, and open field test. Our investigation shows that the forced swim test is less than 50 percent effective at identifying effective human antidepressants when used by pharmaceutical companies in research and development. We will present data describing the discrepant findings in published experiments relying on these tests. We also discuss the continued prevalence of these tests in publicly funded research in the United States and the harms experienced by the animals subjected to them.



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## A novel strategy combining ITS and read-across to predict the skin sensitization potency of chemicals

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The integrated testing strategy (ITS) developed by Kao covers three alternative models, namely h-CLAT, DPRA and DEREK, as a defined approach for the prediction of skin sensitization potency classes ( $EC3 < 2\%$  = Strong;  $EC3 \geq 2\%$  = Weak; Non-sensitizer). To enable a quantitative risk assessment, it is necessary to specify the potency level (EC3) without underestimating the true target potency. To address this issue, we developed a strategy combining the ITS and read-across using relevant analogues with LLNA data. Firstly, read-across was performed to identify suitable analogues of target chemicals using five *in silico* tools (ChemTunes.ToxGPS, ToxRead, AMBIT, OECD TB & TIMES-SS). Secondly, by performing the ITS for the target and analogues and comparing the analogue LLNA data with the ITS result, the potency class of the target was predicted with high confidence. Finally, an EC3 value for the target was predicted by comparing all QSAR and *in vitro* data from the target and its analogues. To determine the applicability of the novel strategy, we performed four case studies using chemicals that were underestimated by the ITS alone, namely Isopropylidenediphenol Diglycidyl Ether (DGEBA), Hexyl Cinnamal, 2-Nitro-p-Phenylenediamine (2-NPPD), and Benzyl Salicylate. By using our novel strategy, the potency class of all target chemicals was predicted correctly as strong or weak skin sensitizer without underprediction. Moreover, for DGEBA ( $EC3 = 1.5\%$ ) and Hexyl Cinnamal ( $EC3 = 10.7\%$ ), the target EC3 values were predicted on the basis of analogue EC3 values ( $EC3(p\text{-tert-Butylphenyl glycidyl ether}) = 0.37\%$  and  $EC3(\text{Amyl Cinnamal}) = 7.6\%$ , respectively). For Benzyl Salicylate ( $EC3 = 2.9\%$ ), the predicted target EC3 value was 2.0%, the lower limit of the potency class “Weak”. These results indicate that our novel strategy enables a conservative prediction of the skin sensitization potency.

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## The evaluation of CHO cell clustering assay to test for pertussis toxin using automatic whole well image capture and analysis

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In recent years, rapid advances based *in vitro* methods and improved imaging analysis have increased the relevance as alternatives to animal-based tests. Therefore, Chinese Hamster Ovary (CHO) cell assay, based on the clustering effect of pertussis toxin (PT) on the cells, has been developed to test for residual PT and reversion to toxicity of acellular pertussis vaccines as one of alternatives to animal-based test. The use of standardized protocol including scoring of the cellular morphologic change by microscopic observation is important to ensure the assay reliability, but there are difficulties with the microscopy and identifying clusters. With this background, we examined the method where we can conduct automatic image capture of whole well of a microtiter plate. This allows us to detect objects and define the PT positive concentration on CHO cells using image analysis.

**Methods:** We prepared PT two-fold dilution series and added them to cultured CHO cells in 24 and 96 well plates. Cell images in the wells were captured 48 hours later and analyzed using the cell imager Cell3iMager duos (SCREEN Holdings Co., Ltd., Kyoto, Japan).

**Results:** We observed that shapes of the cell colonies were round when higher concentrations of PT were added. Therefore, we adopted “circularity” as a colonies’ image characteristic and analyzed their shapes. We observed that the average circularity of the colonies correlated with the PT concentration. These results were reproducible and obtained by the assays using both 24 and 96 well plates. We also applied deep learning methods to detect PT positive and negative colonies, and the results showed good correlations with the PT concentration.

**In conclusion,** this method of the CHO assay has advantages, such as, a) short time for the analysis because of automatic imaging and analysis, b) allowing quantitative measurement instead of binary (positive/negative) or semi-quantitative detection.

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## A validation study of the IATA-based read-across in nephrotoxicity of aminophenols

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Integrated Approaches to Testing and Assessment (IATA) is one of the useful concepts to enhance accuracy of read-across conclusion for systemic toxicity (Sakuratani et al., 2018). However, there are few case studies that have verified how much IATA can contribute to improving accuracy of read-across. Under this circumstance, we developed case studies for hepatotoxicity previously (Nakagawa et al., 2020). Considering the variety of target organs, we focused on nephrotoxicity because the kidney is likely to become the target organ of toxicity following the liver (Vinken et al., 2012) and there are few case studies of read-across for nephrotoxicity.

Aminophenols, which have the potency of nephrotoxicity, were chosen as the target categories in this study. Category compounds including 6 compounds which have *in vivo* data are structurally similar but have different toxic intensities. For example, p-aminophenol and p-methylaminophenol induced clear toxicological effects like renal tubular necrosis *in vivo*, while m-aminophenol only cause brownish pigment. In order to validate the IATA-based read-across predicts those pieces of *in vivo* toxicological information properly, we estimated the adverse outcome pathway (AOP), and then performed *in silico/vitro* evaluation to compare them in the target category. Specifically, we conducted some *in vitro* assays for comparing production of oxidative stress, mitochondrial injury, and renal tubular epithelial cell necrosis, which are the major key events in the AOP of category compounds. These analyses showed that these results of *in vitro* evaluation evaluating cytotoxicity, mitochondrial toxicity and oxidative stress intensity were well correlated with the toxic intensities of compounds in *in vivo* toxicological data.

Thus, this study showed the possibility that IATA-based read-across considering various information such as biological response could estimate differences in toxicity that couldn't be predicted by structural similarity, and enhanced accuracy of the read-across conclusion.

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## 3Rs self-assessment tools to support research groups and institutions to track, evaluate and benchmark their 3Rs activities

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Obtaining a realistic evaluation of 3Rs activities is essential for researchers and organisations seeking to assess or improve their 3Rs culture. The NC3Rs has recently launched two 3Rs self-assessment tools, one for research groups and the other for research institutions. These are free-to-use, secure, interactive, online resources that are designed to allow the user to track, evaluate and benchmark their 3Rs activities over time. Both consist of a series of questions on the 3Rs, developed in consultation with the scientific community and divided into thematic categories representative of the breadth of 3Rs-related activities that can be undertaken by a research group or institution. Once a question set is completed online, the system scores the responses and provides bespoke feedback on how improvements can be made to further support 3Rs implementation.

There are a number of benefits to using the tools. Scores can be used internally to allocate resource and effort, and to focus discussions at research group meetings or in the local ethics committee towards the most important areas and topics. The feedback gives useful advice and examples, helping to ensure that subsequent 3Rs efforts are successful. The 3Rs self-assessment can be repeated periodically and the results compared longitudinally, allowing progress to be tracked. Finally, scores can be used externally (e.g., in grant proposals and licence applications to use animals; or as part of discussions with regulatory bodies or the general public). Overall, use of the self-assessment tools will encourage a more active 3Rs culture and assist in delivering on commitments to the 3Rs.

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## **In vitro antitumor activity of a novel organoselenium compound via transferrin-conjugated PLGA nanoparticles**

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Cancer is one of the major causes of mortality worldwide, and most of the available chemotherapy has many drawbacks because of its non-specificity (Ramzy et al., 2017). Thus, there is a great interest in the development of new drugs as well as new drug delivery systems to achieve a more effective antitumor treatment. In this sense, here we proposed a drug delivery system based on poly(lactic-co-glycolic acid, PLGA) nanoparticles (NPs) as a carrier for a seleno-aminothymidine with prominent antitumor activity (5'-Se-(phenyl)-3-(amino)-thymidine, ACAT-Se) (Rosa et al., 2017). PLGA nanoparticles were formulated with poloxamer as stabilizer (PLGA-NPs), and further surface-modified with transferrin for tumor targeting (PLGA-Tf-NPs). NPs were evaluated for physicochemical properties, showing suitable results. *In vitro* toxicological studies are extremely relevant on the evaluation of NPs, since they provide a rapid and effective mean to assess a number of toxicological endpoints (Arora et al., 2012). Therefore, the *in vitro* hemocompatibility of PLGA-Tf-NPs were assessed by the hemolysis assay. The NPs are non-hemolytic in the tested concentrations after 5 h of incubation (2.80% at the highest concentration, 300 µg/mL). Finally, the antitumor activity of the PLGA-Tf-NPs was evaluated using *in vitro* cell models. The assays were performed for PLGA-Tf-NPs, PLGA-NPs and free ACAT-Se, using tumor cell lines, A375 (human melanoma) and U87 (human glioblastoma), which were exposed for 24 and 72 h to each treatment and their viability was assessed by MTT assay. The results evidenced that PLGA-Tf-NPs significantly improved the *in vitro* antitumor activity of ACAT-Se with respect to the free compound and the PLGA-NPs. Therefore, our results suggest that the developed PLGA-Tf-NPs could be a promising system for a more efficient antitumor therapy. Moreover, the importance and applicability of *in vitro* methods were highlighted as a feasible approach to study the toxicity and biological activity of novel formulations.

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## **In vitro prediction of in vivo pseudo-allergic response via MRGPRX2**

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**Background:** Some compounds administered subcutaneously to rodents cause injection site reactions. In some cases, injection site reactions were caused by a pseudo-allergic response, due to off-target mediated histamine release from mast cells (Petersen et al., 1997). Past practice used post-mortem evaluations of the injections site swelling in rats as correlation for histamine release, thereby enabling selection of drug candidate without pseudo-allergic response.

Literature reports (McNeill et al., 2015; Bulfone-Paus et al., 2017) suggested that activation of MRGPRX2, a receptor expressed on mast cells in the skin, induces a pseudo-allergic response that results in swelling and hyperemia at the injection site. To determine whether the observed injection site reactions were linked to activation of MRGPRX2, the correlation of MRGPRX2 activation *in vitro* to the occurrence and severity of post-mortem injection site swelling was evaluated.

**Results:** Internal *in vitro* studies showed that compounds that caused injection site reactions in rodents also activated MRGPRX2, and that the effect on this receptor correlated with the severity grade of injection site swelling. Consequently, screening plans were adjusted to use the MRGPRX2 *in vitro* assay as a substitute for post-mortem injection site evaluation, thus eliminating the use of rats for initial screening. Approximately 800 rats were saved based on calculation of "worst-case scenario" for the number of compounds tested in the *in vitro* MRGPRX2 assay – where one *in vivo* study consists of n = 4 rats/compound and n = 4 rats for vehicle control group per 4 compounds. Note that use of an *in vitro* assay also allowed for more compounds to be screened resulting in reduced project timelines.

**Conclusion:** A commercially available *in vitro* assay that replaces animal use could be suitable in cases where compounds induce pseudo-allergic reactions. This will prove to be a faster, more ethical tool for the development of safe new pharmacotherapies for patients.



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## 3D spheroids of chorioretinal endothelial cells as an alternative-to-animal model for diabetic retinopathy

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Diabetes Mellitus has been reported as the fifth leading cause of blindness, globally. Approximately, one-third of diabetic individuals develop Diabetic Retinopathy (DR), a vision threatening condition, which progresses from non-proliferative to proliferative stage and eventually culminates into a severe stage of diabetic macular edema. (Pandey et al., 2018; Lee et al., 2015; Lu et al., 2018) Here, the retinal and choroidal endothelial cells are the primary sites of metabolic damage. (Lechner et al., 2017) Existing treatment modalities like laser photocoagulation, pharmacotherapy using corticosteroids and anti-VEGF inhibitors and vitrectomy have exhibited limited effectiveness in clinical settings. Moreover, animal models used for preclinical screening pose significant ethical issues. (Lu et al., 2018; Sripriya et al., 2017) This investigation, thus, proposes the development of an alternative-to-animal, *in-vitro* three-dimensional (3D) model of chorioretinal vasculature to recapitulate the *in-vivo* dynamics of the diseased tissue. The said model was developed using RF/6A chorioretinal endothelial cells and round-bottom ultra-low attachment microplates to facilitate matrix-free generation of endothelial cell spheroids. Viable and proliferating spheroids (mean diameter =  $253 \pm 6 \mu\text{m}$ , mean area =  $50252 \pm 26 \mu\text{m}^2$ ) were developed on day 5 of cell culture, as confirmed by phase contrast microscopy, EZ Blue™ cell proliferation assay, live/dead staining and confocal microscopy. Histological staining demonstrated uniform cellular distribution across the solid microtissue. The resulting model was functionally validated using *in-vitro* sprouting

angiogenesis assay. Changes in spheroidal morphology and effects on cell proliferation were assessed upon individual, as well as concomitant, treatment with Vascular Endothelial Growth Factor (VEGF) and anti-VEGF therapeutic agent, Bevacizumab. These investigations indicated the capability of the developed 3D RF/6A spheroids to act as a pathophysiologically relevant model for the comprehensive understanding of pathophysiology of DR and provide a preclinical platform for developing effective pharmacological interventions for this disease.

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## A novel microfluidic platform for pulmonary nanoparticle exposure

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Nanoparticles (NPs) are a common byproduct of many modern technologies and industrial processes. Studying their toxicity, however, has been a challenge due to the lack of appropriate *in vitro* models. NPs are mainly taken up via inhalation and they deposit deep into the lung. The lung's barrier function determines the uptake of NPs into the system and their resulting toxicity on



other organs. Furthermore, their acute toxicity on the lung tissue needs to be considered.

Our aim was to establish an *in vitro* alveolar lung and liver model for airborne NP testing using a cutting-edge organ-on-a-chip technology. This, in combination with the adapted P.R.I.T.<sup>®</sup> ExpoCube would enable elaborate toxicity studies, where lung equivalents are subjected to repetitive nanoparticle exposure and toxic effects on both lung and liver can be examined.

In this work, we present the development of a hAELVi cell line-based lung model, mimicking the alveolar epithelial cell type I (AECI) and its successful co-cultivation with induced pluripotent stem cell derived liver organoids in a specifically adapted HUMIMIC Chip platform. During the 5-day dynamic cultivation, the hAELVi cells formed a tight barrier, demonstrated by their transepithelial electrical resistance values of up to 3500  $\Omega$ ·cm<sup>2</sup>. Strong expression of the AECI specific and tight junction genes ICAM1, HOPX, CAV1 and ZO1 further confirmed their suitability. Although the liver spheroids experienced major morphological changes during the cultivation, they remained viable and expressed key hepatic markers.

Our results demonstrate an *in vitro* test system compatible with the modified P.R.I.T.<sup>®</sup> ExpoCube to mimic nanoparticle exposure-prone environments for safety assessments, generating high-quality *in vitro* data predictive of nanoparticle safety in humans.

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## Next generation risk assessment of human exposure to anti-androgens using newly defined comparator compound values

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Next Generation Risk Assessment (NGRA) can use the so-called Dietary Comparator Ratio (DCR) to evaluate the safety of a defined exposure to a compound of interest (Dent et al., 2019). The DCR compares the Exposure Activity Ratio (EAR) for the compound of interest, to the EAR of an established safe level of human exposure to a comparator compound with the same putative mode of action. A  $DCR \leq 1$  indicates the exposure evaluated is safe. The present study aimed at defining adequate and

safe comparator compound exposures for evaluation of anti-androgenic effects, using 3,3-diindolylmethane (DIM), from cruciferous vegetables, and the anti-androgenic drug bicalutamide (BIC). EAR values for these comparator compounds were defined using the AR-CALUX assay. The adequacy of the new comparator EAR values was evaluated using PBK modelling and by comparing the generated DCRs of a series of test compound exposures to actual knowledge on their safety regarding *in vivo* anti-androgenicity. Results obtained supported the use of AR-CALUX-based comparator EARs for DCR-based NGRA for putative anti-androgenic compounds. This further validates the DCR approach as an animal free *in silico/in vitro* 3R compliant method in NGRA.

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## Non-animal efficacy testing approaches for ectoparasiticides

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A growing number of efficacy test methods can replace the use of dogs and cats in the assessment of ectoparasiticides. Here we show how *in silico* models and *in vitro* assays can be used in a weight of evidence approach to reduce or replace animal use in efficacy testing of flea and tick control products. For example, we describe artificial membrane systems, highlighting case studies that have evaluated their use. We outline the scientific advantages of animal-free approaches, such as greater control and standardization and the direct observation of the attachment, feeding, and effects of the product on fleas and ticks. We also discuss challenges to regulatory acceptance of these approaches, including the need to further optimize systems for multiple species of ticks, and outline action items to overcome these obstacles. Lastly, we discuss the potential analysis of existing data to identify opportunities that reduce animal use without compromising the evaluation of the efficacy of ectoparasiticides.

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### Use of transcriptomics to substantiate similar biological activity in a read-across exercise for safety assessment of cosmetic ingredients

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We have used *in vitro* transcriptomics to assess the biological activity of structurally related chemicals to define their biological similarity and with that, substantiate the validity of a read across approach usable in risk assessment. We evaluated 4 short alkyl chain parabens: methyl-, ethyl-, butyl-, and propylparaben, as well as their main metabolite, p-Hydroxybenzoic acid (pHBA). The comprehensive transcriptional response of MCF7 cells was evaluated after exposure to vehicle-control, each paraben or pHBA at 3 non-cytotoxic concentrations (1, 50 and 500  $\mu$ M), for 6 h. Differentially expressed genes (FDR  $\geq$  0.05, and fold change  $\pm$  1.2  $\geq$ ) were identified for each of the parabens or pHBA at each dose and used to determine similarities between them. The transcriptional profile elicited by each of the parabens shares a high degree of similarities across the category members. We identified 133 common genes whose expression is modified by each of the parabens in a significant manner in the same direction. pHBA elicited significant gene expression changes at the highest concentration evaluated (615 genes), however, these changes are mostly different than the ones elicited by any of the parabens. The highest number of genes commonly affected by the parabens was found between butylparaben and propylparaben, where 634 genes were in common. Pathway enrichment analysis (MSigDB v7.0) of the transcriptional profile for each paraben indicated a significant overlap in the up- and down-regulated pathways across the four parabens. The highest similarity in biological activity was found between butylparaben and propyl-

paraben. The top Hallmark pathways that are most up-regulated by these two parabens are: estrogen response early and late, and TNFA signaling via NFKB. While the most down-regulated are: apical junction, NOTCH and hedgehog signaling. These pathways' similarities further support the conclusion that these two parabens are the most similar structural and biological analogs. Supported by Cosmetics Europe.

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### RAINBOWFLOW CHIP: An impedance-based biosensor for chemical hazard assessment with fish cell lines at its core

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Fish are important indicator species for contamination of the aquatic environment, thus millions of fish are sacrificed annually in toxicity experiments for chemical hazard assessment and effluent testing (Scholz et al., 2013). One alternative is the use of fish cell lines derived from rainbow trout, which have been employed in a variety of *in vitro* assays to determine, e.g., acute toxicity of chemicals (Schirmer, 2006). Recently, bio-impedance monitoring of fish cell lines was established to follow the acute toxic response over time. Herein, cells are seeded on an electrode chip and resistance is measured non-invasively, reflecting the health status of the cells. A decrease in resistance is an indicator for loss of cell viability as can be elicited, for example, by exposure to chemicals (Tan and Schirmer, 2017). In the RAINBOWFLOW CHIP project, we employ this technique to establish time-resolved analysis of cell viability in response to chemical exposure under flow conditions. For this, we use an intestinal cell line of the rainbow trout (*Oncorhynchus mykiss*) – RTgutGC – which, similarly to the gill cell line, RTgill-W1 (Tanneberger et al., 2013), shows good correlation with fish *in vivo* LC50 results from acute toxicity tests (Schug et al., 2020). Yet, adherence of RTgutGC cells is superior to the RTgill-W1 cells, which is beneficial for use in impedance sensing chips. Moreover, while the gill is the first site of contact and primary uptake site for water-borne chemicals, the gut is the first tissue of contact for toxicants adhering to organic matter (including food), as is the case for hydrophobic chemicals. In the RAINBOWFLOW CHIP, the



flow-through design leads to constant replenishment of the test substance, thus providing stable exposure concentrations, which is especially important for difficult-to-test (i.e., volatile and hydrophobic) chemicals that experience losses in the conventional static test set-up.

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## Verification of the applicable domain of reactive oxygen species (ROS) assay for developing photosafety cosmetic ingredients

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A non-animal testing approach was proposed for evaluating photosafety of cosmetic ingredients using *in vitro* photo-reactivity assays; however, complex cosmetic ingredients, such as plant extracts and polymers, could not be evaluated because their molecular weight (Mw) is often poorly defined. A reactive oxygen species (ROS) assay was successfully accepted as OECD Test Guideline (TG) No. 495 for evaluating photo-reactivity of Mw defined chemicals (OECD, 2019). ROS assay evaluation at the weight concentration (50 µg/mL) was reported in our previous paper (Nishida et al., 2015), however the rationale for setting concentration and its verification were insufficient. Therefore, in this study, ROS assay was performed after setting tentative molecular weights (tMw) and the applicability was verified from the viewpoint of the sensitivity in cosmetic ingredients.

Twenty-one plants oils and forty-four molecular weight defined chemicals were selected as test chemicals. The tMw was defined as 150, 200, 250, 300 and 350 g/mol, respectively and

then their photo-reactivity was assessed in TG No. 495. Setting the tMw to 250, photo-reactivity can be evaluated with 100% sensitivity, and specificity was relatively high compared with other conditions. The molecular weight defined chemicals were also evaluate with 100% sensitivity in the tMw to 250. It was indicated that the applicability to unknown substances was in the tMw to 250. In addition, the photo-reactivity of plant extract, polymer, and other cosmetic ingredients were also evaluated.

The sensitivity of ROS assay is significantly high and test chemicals found to be negative are likely to be no concern about the photo-reactivity. Cosmetic ingredients which molecular weight cannot be clearly defined are difficult to evaluate the photosafety using the Mw-based test method. ROS assay is likely to be useful as photo-reactivity evaluation approach for Mw unknown chemicals. It was expected that ROS assay contributed to the development of highly photosafety ingredients.

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## Animal dissection in teaching minors (under 19 years of age) in Korea – Contradictory Korean legislation and enforcement rule

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Effective 21 March 2020, the practice of animal dissection, including using dead animals for the teaching of minors (a person under 19 years of age), is prohibited in Korea under the Animal Protection Act (Article 24-2 Prohibition of Minors' Animal Dissection Practice). However, a recent revision of the Enforcement Rule of the Animal Protection Act (enforcement date 21 March 2021) inserted a new Article 23-2 (Exception to Prohibition of Minors' Animal Dissection Practice) and detailed guidance (Addendum 5-2, Ethical review committee SOP on



animal dissection practice); which provides substantial exclusions that allow for the continued practice of dissection. The new Enforcement Rule apparently reverses most of the prohibition against using animals in the teaching of minors. If there is no compelling reason under Article 23-2-2-4, the practice of animal dissection by minors is allowed in school under the Elementary and Secondary Education Act and Gifted and Talented Education Act, or a research institution prescribed by the Ordinance of the Ministry of Agriculture, Food and Rural Affairs. In contrast, the promotion and protection of research subjects – including animals – are among the core competencies of well-educated involved personnel. The Animal and Plant Quarantine Agency even published the “Guidelines for Animal Practice in Elementary, Middle, and High Schools” in December 2019. Since 2005, the presenting author has researched the 3Rs alternatives to animal use in education and have provided a platform for empowering teachers to find humane substitutes for animal dissection since 2008. The Korea Information Center for the 3Rs (KIC3Rs), established in 2011 in collaboration with three national and five global organizations, provides a framework, suggesting Animalearn’s “The Science Bank” as an appropriate model to follow. It is time to establish an advanced platform that enables more dynamic and interactive learning experiences and resources through global collaboration reflecting Korean culture.

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## Optimization of gene silencing in a cell-laden 3D organ-like model by means of RNA interference

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Three-dimensional (3D) cell cultures which mimic the complex environment and natural physiological conditions in native tissues, not only provide a better model for *in vitro* responses in comparison to the conventional monolayer two-dimensional (2D) cell cultures but can also eliminate the need for animal models. Hiller et al. (2018) and Berg et al. (2019) have shown the potential of bioprinted 3D tissue models for viral infection and viral gene delivery studies. However, suitability of cell-laden 3D organ-like structures for non-viral gene delivery as a safe alternative to viral vectors has not yet been fully realized. The goal of this study was the optimization of the non-viral gene delivery and gene silencing in 3D cell-laden constructs using RNA interference (RNAi). For this purpose, alginate-based 3D constructs, supplemented with Matrigel (20%), containing HEK293T cells were generated using extrusion printing. The 3D structures were transfected with siRNA against human cyclophilin B (hCycB), a cyanine-labelled (Cy3)-siRNA, or a green fluorescent protein (GFP)-encoding plasmid, with different transfection reagents (Isifect and Lipofectamin 2000). Subsequently, RNAi-mediated silencing by transfection with hCycB siRNA was examined by quantitative polymerase chain reaction (qPCR). In addition, the expression of GFP as well as the fluorescence of the Cy3-tagged siRNA within the generated 3D constructs were analyzed by fluorescence microscopy. The results of this examination showed a distinct hCycB gene silencing in 3D construct transfected with both Isifect and Lipofectamin 2000. Moreover, in order to improve the transfection, the 3D constructs were modified to reduce the alginate-induced cell encapsulation within the 3D constructs. For this purpose, the 3D constructs were treated with sodium citrate, which partially degraded the alginate cross-links within the constructs. The subsequent fluorescence microscopic examinations and qPCR analysis showed that the addition of sodium citrate favored the transfection of the generated 3D constructs.

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## Systematic review protocol on the effect of fecal microbiota transplantation on behavior in animals

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The gastrointestinal tract harbors a complex ecosystem comprised of trillions of microorganisms which have significant local and extra-intestinal effects on an organism's health. The bi-directional interaction between the gut microbiota and the central nervous system has been coined the gut microbiota-brain axis. While growing evidence suggests the intestinal microbiome can affect an organism's behavior, to show causality and determine the extent of the contribution of the microbiota, fecal microbiota transplantation (FMT) must be performed. FMT is the administration of a solution of fecal matter from a donor into the intestinal tract of a recipient. Behavioral change in FMT recipients provides crucial evidence for the gut microbiota-brain axis and its influence on behavior. Therefore, our group initiated a systematic review (SR) to evaluate the evidence that animal behavior can be affected by FMT. An SR protocol was developed and published on Oct 21, 2019 on the SYRF (CAMARADES/NC3Rs Systematic Review Facility) online platform (<http://syrf.org.uk/protocols/>). Using the search strategy detailed in the protocol, a search of PubMed and Embase databases yielded 13,160 unique references. Two independent reviewers performed title and abstract screening and identified 552 references for the full text screening phase. Full text screening is now complete, and 86 references have been selected for data extraction. Our SR will give a qualitative overview of the use of FMT in behavioral animal studies, will investigate the prevalence of pseudoreplication, and aims to identify areas for improving experimental design needed to prove or disprove the role of the intestinal microbiota in modulating behavioral outcome measures. By identifying ways to improve the internal/external validity and reproducibility of FMT animal studies, less animal experiments need to be performed and clinical translation of preclinical animal studies will be improved.

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## SkinEthic™ HCE Time-to-Toxicity: The first individual method formally considered by the OECD for discriminating on its own the three UN GHS ocular hazard categories

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For more than two decades, scientists have been trying to replace *in vivo* rabbit eye irritation test with non-animal methods. So far, several *in vitro* methods have been implemented into regulations, however none of them is able to replace the test completely due to the complexity of the endpoint and the classification schemes applied by the regulation under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS). Taking into account our expertise on the SkinEthic™ HCE model (test system of OECD TG 492) and our knowledge on associated protocols, the SkinEthic™ HCE Time-to-Toxicity test method was established.

The test method evaluates the hazard potential of a chemical based on its ability to induce cytotoxicity. Based on the viability observed for the different exposure periods (from 5 to 120-min) a classification was assigned.

In the current study, the method was developed with 74 training chemicals (32 liquids, 42 solids) and challenged with 52 test chemicals (24 + 28) selected on the basis of important *in vivo* drivers of classification (i.e., corneal, conjunctival and persistence effects).

The within laboratory reproducibility (concordance within 3 runs), based on 67 training and 30 test chemicals was 87% independently of the testing sets.

Application to 74 training chemicals, accuracy value was above 72%, with 75% Cat. 1 (N = 25), 68% Cat. 2 (N = 20) and 74.9% No Cat. (N = 29) correctly identified. Furthermore, 80% Cat. 1, 71% Cat. 2 and 70% No Cat. were well classified with the test set, confirming the robustness of the test method. In conclusion, this study provides evidence that the SkinEthic™ HCE Time-to-Toxicity method is capable of i) correctly identifying chemicals not requiring classification AND ii) distinguishing between Cat. 1 (serious eye damage) and Cat. 2 (eye irritation), therefore providing added value for the classification and labelling of chemicals according to the three UN GHS.

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## ReThink3R – Design Thinking Workshops towards the implementation of the 3Rs

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The combination of numerous obligations, time constraints and inflexible research structures often leaves little room for early career scientists performing animal experiments, to address the 3R topic and ethical concerns in a satisfying manner. Moreover, scientists need to be empowered to be innovators in their field through training of 21<sup>st</sup> century skills, which include critical thinking, collaboration, creativity and empathy. In order to overcome this predicament, we are driven to offer interested scientists the possibility of dealing with the implementation of the 3Rs in various contexts through interactive Design Thinking Workshops (“ReThink3R”). Design Thinking is an innovation method that combines both analytical and creative methods to find user-centered solutions in an iterative process. Our workshop usually begins with a challenge, such as “Design an institute that keeps animal suffering to a minimum”. During the first exercises, the participants perform interviews with different stakeholders. Thus, they get to know different perspectives and necessary information to uncover concrete underlying problems. Issues here-in were for example i) time limitations due to excessive bureaucracy, ii) insecurities in animal handling and iii) a lack of knowledge or trust in alternative methods. All gathered information and insights are used in the solution process and shape the following generation of ideas, out of which one is developed further through quick prototyping and testing with users for validation. Here, we will present the results of the 14 workshops that have been performed so far at different graduate schools and institutions in Berlin and the University of Zurich. This new workshop approach guides scientists through a difficult and emotionally complex topic and aims at training scientists in teamwork, an open-mindset and creative confidence – facilitating a change within the scientific community including awareness for the 3Rs.

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## Cosmetics Europe eye program: Application of two defined approaches for ocular toxicity predictions based on *in vitro* bottom-up approach on 4 case studies

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The eye irritation/severe eye damage historically played a key role in the development, validation and regulatory acceptance of *in vitro* test methods and remains an active topic for regulatory toxicology, currently within the frame of the Organisation for Economic Cooperation and Development (OECD), Test Guideline Program. Recent activities in developing defined approaches (DAs) and how those fit have been achieved for liquids chemicals. From the scientific side, the DAs allow to perform predictions over the entire spectrum of United Nations Global Harmonized System for classification and labelling (UN GHS Categories 1, 2 and No Category), by strategically combining physico-chemical properties to sequential *in vitro* testing on Reconstructed human Cornea-like Epithelium (RhCE) and Bovine Corneal Opacity and Permeability Laser Light-Based Opacimeter (BCOP LLBO) in a first DA bottom-up approach; and BCOP LLBO and short time exposure (STE) test in a second DA bottom-up approach.

In the present work, application of both DAs is exemplified with specific case studies on four chemicals. Among the tested chemicals, a chemical (1,3-di-isopropyl benzene) known to be No Cat. *in vivo*, is predicted as No Cat. in the first tier strategy test methods of the DAs. Concerning the positive calls on the 2 known *in vivo* Cat. 2 chemicals (2-Ethyl-1-Hexanol and 2-Methyl-1-Pentanol) and an *in vivo* Cat. 1 chemical (2-Hydroxy-Isobutyric acid ethyl ester), the second-tier step which consists of their evaluation in the BCOP LLBO test which sub categorized them as Cat 2 / Cat. 1, respectively. In conclusion, these case studies reflect 2 approaches on how to move from animal testing into an evaluation of new ingredients based on examples of application of DAs on an Integrated Approach for Testing and Assessment for safety purposes of ingredients.

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## The botanical safety consortium's strategy for developing a robust framework of genotoxicity assays for safety assessment of botanical substances

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Botanical substances have been widely used for centuries in efforts to preserve and enhance human health and well-being. Safety evaluations of these substances are typically based on adverse event reports, historical textbook knowledge, and other published information, either scientific or anecdotal. For ingredients with insufficient safety data, animal models have often been used to investigate potential health risks prior to testing in a clinical setting. Today, a pressing need exists to define the appropriate levels of chemical characterization, identity standards, and safety evaluation required to support the safe use of these complex botanical substances. The Botanical Safety Consortium (BSC) is addressing this need by organizing an international effort to bring together scientific experts to create a botanical safety toolkit for botanical dietary ingredient assessments. The toxicological assessment must include an evaluation of genotoxicity potential, as genotoxicity is associated with a number of adverse human health effects that are not reliably predicted by spontaneous adverse event reporting. Established *in vitro* and *in silico* test methods are available and can support assessment of the genotoxicity of botanical ingredients. The BSC's Genotoxicity Technical Working Group (TWG) is developing a pragmatic fit-for-purpose testing strategy for botanical ingredients. The BSC's analytical and data analysis TWGs will support the Genotoxicity TWG in the characterization of samples of interest. A testing strategy will be developed, including the use of high-throughput screening technologies for the identification of potentially important chem-

ical constituents in products already being marketed. The goal of the BSC is to develop predictive toxicology testing strategies for botanicals by integrating existing published data and the identified *in silico* and *in vitro* tools into a robust, comprehensive program that provides actionable safety data while minimizing the need for animal testing.

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## Search for candidate miRNAs implicated in putative adverse outcome pathway (AOP) relevant to Alzheimer's disease

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Sporadic Alzheimer's disease (sAD) is a progressive neurodegenerative age-related disease, caused by interaction of genetic and environmental factors, leading to brain damage accompanied by memory loss and cognitive impairment. Up till now, animal-based approaches developed to elucidate the AD-related pathophysiological mechanisms, failed to translate into effective therapeutic treatments or tools for diagnosis. In addition, existing research on AD development is mainly focused on the genetic (familial) type of AD with the most extensively studied AD-related mechanism being, amyloidopathy and tauopathy. The initiating events, needed for detection of early AD pathogenesis, remain elusive. Subsequently, the diagnosis of sAD at early stage is currently poor and inaccurate.

During the last decades, microRNAs (miRNAs) have attracted much attention due to their fundamental role in the modulation of numerous biological processes. Several human miRNAs have been suggested as promising biomarkers for neurotoxicity and AD development. Systematic literature searches revealed processes shared by neurotoxic compounds and sAD, suggesting that established novel approach methods (NAMs) developed for toxicity testing may provide insight in the early processes of sAD development.

The existing human and animal data, as well as NAMs data were structured using the adverse outcome pathways (AOPs) concept. While developed for toxicology, the AOP concept was proven to be a useful tool for collecting complex biological knowledge on diseases. Proposed AOPs for AD pathogenesis may improve the understanding of the potential chain of events, triggered by molecular initiating events and linked to adverse outcomes. In this perspective, the identification of miRNAs functioning these suggested sequential events by known AOPs



for neurotoxicity or putative AOPs for AD development, may support the discovery of predictive biomarkers commonly dysregulated in both neurotoxicity and AD. Taken together, the existing data of miRNAs may provide an effective approach to understand the mechanisms underlying the AD development, even at early AD stage.

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### Improving quality of *in vitro* methods: A GIVIMP certification program

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Recently the Organization for Economic Cooperation and Development (OECD) issued a Guidance Document on Good In Vitro method Practices (GIVIMP). Intended to reduce the uncertainties in cell and tissue-based methods used in the prediction of human safety, GIVIMP provides the field with a set of standards to improve both the quality and accuracy of newly developed, and routinely executed, *in vitro* methods. Currently organizations are struggling to define the best approach for practical implementation of the guidance within their programs. As GIVIMP is broad in scope and covers a wide range of scientific and quality topics, there is the potential for varying interpretations of the guidance and thus significant differences in implementation. A business-to-business certification program is one solution to harmonize GIVIMP interpretation, standardize “claims” of compliance with the document, and provide a uniform roadmap for incorporation of GIVIMP principles within routine laboratory operations and method development activities. A certification program would be beneficial to the full audience for which GIVIMP was intended including academic laboratories developing new methods, established laboratories participating in validations and/or performing routine *in vitro* studies, and industry laboratories intending to submit *in vitro* data to regulatory agencies. A pilot certification between the Institute for In Vitro Sciences (IIVS) and BASF SE laboratories (Ludwigshafen, DE) has been launched to provide proof-of-concept for the program. This poster discusses the need for the GIVIMP certification program and provides details on its structure and administration.

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### Population effect on drug and metabolism absorption using *ex vivo* intestinal tissue explants in the Intestine Explant Barrier Chip

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Poor prediction of bioavailability and intestinal drug metabolism hamper drug development and underscore the need for novel predictive models. Additionally, the presence of the microbiota is often lacking despite having an important role in the metabolism of xenobiotics. The Intestine Explant Barrier Chip (IEBC) integrates small sizes of (human or porcine) intestinal tissue using a 3D clicking mechanism, allowing determination of intestinal drug absorption and metabolism. Population effects were studied by exposing the apical side of the intestinal tissue to fecal water containing microbial excretion products (i-screen technology) from healthy donors stratified by age or IBD patients. From a selection of low to high permeability drugs, 6 out of 7 properly ranked according to their *in vivo* fraction absorbed, with a close similarity between porcine and human colon tissue. Low FITC-dextran 4000 kDa permeability confirmed intact tissue barrier integrity and comparable levels of secreted LDH affirmed that none of the drugs showed adverse effects on the tissue. When exposing human colon tissue to fecal water containing microbiome excretion products (i-screen supernatant) from healthy adults or IBD patients treated with the anti-inflammatory drug sulfasalazine, tissue functionality, viability and integrity were not affected. However, lower concentrations of the sulfasalazine metabolite sulfapyridine were detected in the basolateral medium when the tissue was exposed to IBD i-screen supernatant. Furthermore, while the release of pro-inflammatory cytokines IL-6 and IL-8 was dampened when tissue was exposed to sulfasalazine-treated i-screen supernatant of healthy adults, no decrease was observed upon incubation with sulfasalazine-treated IBD i-screen supernatant, potentially illustrating the dysbiotic state of IBD patients' microbiota.

In conclusion, The IEBC enables studying drug absorption and metabolism in human or porcine intestinal tissue explants under physiological conditions and permits determining population effects in combination with microbiome supernatant.



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## Kidney-on-a-chip – Integrating glomerular filtration and tubular reabsorption models

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The kidney's excretory function is crucial in drug development, as it dictates drug clearance and reabsorption. Furthermore, nephrotoxicity of candidate drugs is one of the major reasons for drug attrition. Therefore, an accurate kidney model for Multi-Organ-Chip applications could revolutionize drug trials by providing a relevant *in vitro* platform. Emulating the kidney's distinct functions accurately, however, requires a two-fold approach combining glomerular filtration and tubular reabsorption. The HUMIMIC Chip4 harbors a glomerular and a tubular compartment, which together form the interface between the separate surrogate blood circuit and the excretory circuit. The surrogate blood circuit comprises three additional organ compartments, which could, e.g., contain liver and intestine equivalents for ADME studies.

In this study, iPSC-derived glomerular and tubular organ equivalents are generated and co-cultured in the Chip4. Using iPSC-derived organ equivalents allows the creation of an autologous chip, which has major implications for future studies, as it enables the incorporation of an immune system equivalent. The employed podocytes exhibit typical podocyte morphology and express key markers. For the tubular model, kidney organoids are employed. These organoids contain mostly proximal and distal tubule epithelial cells and few podocytes, endothelial and interstitial cells, as confirmed by immunohistochemistry staining and gene expression analysis. When dissociated and seeded onto a permeable membrane, the cells form a barrier. The developed glomerular and tubular models can be co-cultured within the HUMIMIC Chip4 for several weeks whilst maintaining their morphology and marker expression.

Taken all together, the developed kidney-on-a-chip constitutes a potent tool for advanced *in vitro* drug trials. It is designed to generate high-quality *in vitro* data predictive of renal drug clearance, reabsorption and nephrotoxicity in humans.

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## Assessing the reproducibility of published physiologically based kinetic models

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In order for any chemical (pharmaceutical, cosmetic, environmental pollutant, etc.) to elicit an effect on the body it must possess inherent activity and reach the site of action in sufficient concentration. Physiologically based kinetic (PBK) models can be used to describe the concentration-time profile of a chemical at relevant sites within the body, they are flexible and adaptable to different species, life stages, routes of exposure and dosing scenarios. Historically, generating data for PBK models was intensive in animal use, leading to a drive to develop alternatives methods. One important method to reduce testing is leveraging existing data and applying this knowledge to other chemicals, species, exposure scenarios etc. Many PBK models have been published in the literature for a range of chemicals; in order to apply the knowledge from these models, it is first essential to be able to reproduce the model. To date, there has been little consistency in the way in which such models are recorded and considerable variation in the level of detail supplied. In this study the usefulness and accessibility of three software packages (MATLAB, PK-Sim, and QIVIVE) were used in assessing the reproducibility of published PBK models. Factors such as the model description and interpretation of parameters from the published models, as well as usability and flexibility of the modelling software used to reconstruct the model were all considered. Several issues were identified regarding the model information provided in the published model descriptions and recommendations are given to assist future model development and reporting, particularly with respect to the potential of using such models as templates for other chemicals of interest.



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## Nutritional requirements of fish cell lines – Developments towards a serum-free culture medium

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Permanent fish cell lines of rainbow trout (*Oncorhynchus mykiss*) have great potential as alternatives to conventional fish tests in chemical safety testing (Schirmer, 2006; Tanneberger et al., 2013). While several strategies and assay procedures are being developed that use fully defined media for chemical exposure, the routine culture of these cells, and certain chemical exposure assays, still require fetal calf serum (FBS) for cell maintenance and proliferation (Fischer et al., 2019).

On this background, we aim to develop a fully transparent serum-free cell culture medium for cell lines from rainbow trout, following the media pyramid proposed for optimizing chemically defined media for mammalian cells by van der Valk et al. (2010). According to this pyramid, we will use Leibovitz' L-15 medium as the base. This is a widely accepted basal medium for fish cell cultures and very well suits common cold water fish cell culture conditions, i.e., culture in normal atmosphere and  $\leq 20^{\circ}\text{C}$ . Next, we are gathering available knowledge of the nutritional and hormonal requirements of fish and fish cells in culture to identify suitable supplements. A screening approach that quantifies impact of selected supplements, alone and in combination, on fish cell proliferation and morphology over five days will be used to identify the optimal composition of the supplement.

If successful, this research will overcome the last stumbling block to making fish cell line assays as alternatives or supplements to fish tests in chemical risk assessment truly animal-free. It will moreover further promote the use of fish cell lines beyond toxicology, namely in areas such as fish physiology, pathology and nutrition.

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## Use of 3D human liver microtissues to assess hepatotoxicity of biologics

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Human 3D Liver Microtissues (hLiMT) consist of primary liver cells and preserve liver specific functions and metabolic activity over several weeks in culture. They are an attractive tool for *in vitro* drug safety assessment. hLiMT outperform 2D primary hepatocytes in prediction of hepatotoxicants causing drug-induced liver injury (DILI). However, biologics have never been tested in this system. Here, we assessed the capability of hLiMT to discriminate between interferon (IFN)-lambda (clinically associated with hepatotoxicity) and IFN-alpha2a (approved, no hepatotoxicity). Aspartate aminotransferase (AST), a clinical DILI biomarker previously not used *in vitro*, was combined with the established *in vitro* toxicity markers lactate dehydrogenase (LDH) and intracellular ATP. Upon validating the system with a range of small molecule DILI reference compounds, individual hLiMT were exposed to IFN-lambda and IFN-alpha2a for 7 days with 3 compound additions. Measurement of all AST, LDH, and ATP was performed for each microtissue in a multiplexed design.

The *in vitro* system could show hepatotoxic effects IFN-lambda while IFN-alpha2a had no effect mimicking the clinical profile of both biologics. Changes in ATP level correlated well with AST and LDH signals. The addition of LDH and AST to the commonly used toxicity biomarker ATP allowed for determination of toxicity kinetics. These results show that hLiMT have a great potential for *in vitro* assessment of hepatotoxic biologics and to get first insights into the mechanism of toxicity. We pro-



pose to incorporate *in vitro* testing using hLiMT into the routine preclinical safety assessment for small molecules and selected biologics.

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## Disruption of cellular migration/adhesion as common key event in drug-induced liver injury; options for new *in vitro* testing strategies

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Drug-induced liver injury (DILI) has a poorly understood pathogenesis, diversity of clinical presentations and often idiosyncratic nature. It is therefore, that DILI challenges the AOP-concept to provide relevant key event (KE)-oriented *in vitro* test methods. This challenge has been investigated in the MIP-DILI project, between 2012 and 2017.

Previously, we have found indications that some DILI-inducing pharmaceuticals may be inhibit leukocyte migration motility (trovafloxacin > difloxacin > carbenoxolon) as well as ROCK/MLCK-mediated bile canaliculi (BC) deformations (also TVX, DF). We hypothesize that these important biological processes represent common key events in development of both cholestatic hepatitis and immune-related DILI.

We have set out to investigate the effects of a series pharmaceuticals, known to affect BC dynamics in HepRG cultures, on HL60 neutrophilic cells, RAW macrophages, Cho hamster cells. Among others, we have tested chlorpromazine, cyclosporine A, bosentan, and troglitazone flucloxacillin (all highly active in BC deformation), diclofenac, and trovafloxacin (both less active in BC deformation). We have used, e.g., X-celligence impedance technology to determine concentration-dependent effects on migration and adhesion of these cells. We also tested these drugs for NFkB-activation in tagged THP1-human monocytes.

Data shows that ranking of pharmaceuticals according to the extent of BC deformation matches the ranking according to inhibition of migration (HL60 cells, RAW macrophages) and or adhesion (Cho cells). We additionally confirm that TVX inhibits NFkB activity, but that most other pharmaceuticals that affect BC deformation do not. Importantly, migration of leukocytes has been identified as a central KE in the AOP for inflammation.

Our data supports this idea that, e.g., cellular adhesion, migration, and contraction may represent a common KE of DILI. *In vitro* systems to test new drug entities for this KE may therefore well fit the proposed fit-for purpose tiered approach for DILI, developed in the MIP-DILI project.

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## Use of a dynamic skin and liver co-culture model to investigate the effect of application route on the metabolism of the hair dye, 4-amino-2-hydroxytoluene

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The Cosmetics Europe's Long Range Science Strategy (LRSS) aims to establish the link between dermal and systemic exposure route. In this project, we have used TissUse's microfluidic platform, the HUMIMIC Chip2 model, incorporating skin (EpiDerm™) and liver organoids (consisting of HepaRG™ and stellate cells), to investigate the influence of exposure scenarios on the bioavailability and metabolic fate of chemicals. The aromatic amine hair dye, 4-amino-2-hydroxytoluene (AHT), was selected as a case study chemical to determine whether the Chip2 model could be used to mimic the first-pass effect in the skin that was observed in *in vivo* studies in rats. Both organoids were well maintained over 6 days, as indicated by TEER, metabolic anal-



ysis and viability markers. The kinetics of AHT and several of its metabolites, including N-acetyl-AHT and AHT-sulfate, differed between topical and systemic application. Importantly, topical application resulted in a higher peak concentration of N-acetyl-AHT and increase of its area under the curve (AUC) by 275%, demonstrating that a first-pass effect of N-acetylation in the skin had occurred. There was a concomitant decrease in the peak concentration and AUC of AHT-sulfate after topical compared to systemic application. These results were in accordance with *in vivo* observations, where the ratios of these two metabolites were altered by the application route. In conclusion, these data demonstrate that the Chip2 maintains the functions of skin and liver organoids for several days. Importantly, the Chip2 model recapitulated the route-specific alteration in the metabolite profile of AHT observed *in vivo*. This type of information is important for the risk assessment of topically-applied compounds which may also undergo first-pass metabolism in the skin and whose systemic effects are altered accordingly.

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## Updated dermal sensitisation thresholds derived using an *in silico* expert system and an expanded local lymph node assay dataset

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When conducting a Quantitative Risk Assessment of a potential skin sensitiser, one available tool is the Dermal Sensitisation Threshold (DST). The DSTs are thresholds of toxicological concern representing worst-case scenarios based on the known potency of nearly 300 skin sensitisers. Two values have previously been published: a non-reactive DST of 900 µg/cm<sup>2</sup> based on Local Lymph Node Assay (LLNA) data for 38 sensitisers judged to be non-reactive by human experts (Safford et al., 2011), and a reactive DST of 64 µg/cm<sup>2</sup> based on LLNA data for 233 reactive sensitisers (Safford et al., 2015). This study sought to update these DST values using an expanded dataset, and to investigate assigning chemical reactivity using an *in silico* tool.

An LLNA dataset containing 1,169 chemicals was curated in-house and skin sensitisation structural alerts within Derek Nexus, an *in silico* expert system for predicting toxicity, were used to assign each chemical as reactive or non-reactive. A gamma distribution was fitted to the EC3 values of the 477 reactive sensitisers

to calculate an updated reactive DST, and similarly the EC3 values of the 81 non-reactive sensitisers were used to derive an updated non-reactive DST.

The updated reactive DST was very similar to the original value despite being based on almost twice as many chemicals, highlighting the robustness of this threshold. However, the updated non-reactive DST, also based on twice as many chemicals, was slightly smaller than the original value. A review of the 4 most potent non-reactive sensitisers (with an EC3 value less than 900 µg/cm<sup>2</sup>) revealed two chemicals which were likely to be non-sensitisers containing potent sensitising impurities, and two uncommon toxicophores which are not currently covered by the alerts in Derek Nexus. The dataset is being analysed further with the aim of making the updated DSTs as robust as possible.

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## Next generation risk assessment approach for inhalation exposures: Polymer case studies

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Next Generation Risk Assessment (NGRA) is an exposure and hypothesis-driven approach that integrates new approach methodologies (NAMs) to assure human safety without animal data. This work focuses on the development of an NGRA approach for inhalation exposures using hypothetical case studies of a film-forming polymer in personal care products (e.g., antiperspirants) and a silane in cleaning products.

Impairment of mucociliary clearance, lung fibrosis and lung surfactant inhibition were identified as the relevant endpoints for the most common consumer exposure scenarios (e.g., daily use of an antiperspirant). To investigate these endpoints, two cell models were selected for *in vitro* testing: the MucilAir™-HF cell model (Epithelix) and the EpiAlveolar™ cell model (MatTek). In addition to the two case study chemicals another 16 benchmark chemicals were selected either due to their well-known effects in the specific areas of the lung, history of safe use and/or due



to chemical or physical similarities to the case study chemicals.

Consumer habits and practises were used to derive an airborne concentration ( $\text{mg}/\text{m}^3$ ) for each chemical and exposure scenario, which was then transformed into deposited mass in the bronchial and alveolar region ( $\mu\text{g}/\text{cm}^2$ ) using MPPDv2.8. Cells were then exposed to the predicted concentrations that reflected daily realistic exposures for up to 12 days and different endpoints (e.g., TEER, LDH, cytokines, histology, cilia beating frequency, mucociliary clearance, mitochondrial toxicity, etc.) were measured at 4 different timepoints. Preliminary results indicate that the alveolar model was more sensitive to some of the pro-inflammatory benchmark substances tested. Polyhexamethylene guanidine phosphate for example induced a mild inflammatory response in the MucilAir™-HF system over the 12 days' treatment while inducing significant cytotoxicity in the EpiAlveolar™ cell model after only 4 days of exposure.

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## Identifying and characterizing stress pathways of concern for consumer safety in next generation risk assessment

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Next Generation Risk Assessment (NGRA) is defined as an exposure-led, hypothesis-driven risk assessment approach that integrates New Approach Methodologies (NAMs) to assure safety without animal testing. Within NGRA there is an ongoing need to develop robust and relevant assays that can be used to characterize bioactivity of chemicals at human-relevant exposures (Dent et al., 2018). This type of approach does not aim to identify a specific adverse outcome or pathology but rather aims to be protective of human health by estimating an exposure at which no biological response is expected (Friedman et al., 2019; Wetmore et al., 2015).

The objective of this work was to develop and evaluate a cellular stress response panel that could form part of an early tier testing strategy. This panel consisted of 36 biomarkers representing mitochondrial toxicity, cell stress and cell health, measured predominantly using high content imaging (Hatherell et al., 2020). To evaluate the suitability of the panel for NGRA, data were generated using two sets of benchmark chemicals: chemicals that at defined human exposures are known to cause adverse systemic effects due to cellular stress in a proportion of exposed individu-

als; 2) chemicals that at relevant human exposures have not been associated with adverse systemic effects related to cellular stress.

A Bayesian model was developed to quantify the evidence for a biological response, and if present, a credibility range for the estimated point of departure (PoD) was determined. PoDs were compared with the plasma C<sub>max</sub> associated with the typical substance exposures and indicated a clear differentiation between “low” risk and “high” risk chemical exposure scenarios.

The results presented in this work show that the cellular stress panel can be used, together with other new approach methodologies, to identify chemical exposures that are protective of consumer health.

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## Proposal of a new applicability domain of Vitrigel-EIT (eye irritancy test) method utilizing the pH level and light absorbance of test chemical preparations

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**Introduction:** The Vitrigel-EIT method is a test method that determines the presence or absence of eye irritation with high sensitivity by analyzing the time-dependent changes of trans-epithelial electrical resistance values for 3 minutes after exposing a test chemical preparation (i.e., solution or suspension) to a human corneal epithelium model fabricated in a collagen vitrigel mem-



brane chamber and was registered as TG494 of OECD in 2019. However, acidic chemicals that have a pH level of 5 or less in test chemical preparations and solids are excluded from the applicability domain. In this study, we aimed to propose a revised test method available for test chemicals including solid and established a new test procedure utilizing not the applicability domains for test chemicals but the pretests for test chemical preparations. In the pretests, test chemical preparations showing pH  $\leq 5$  and brief phase-separation within three minutes as much as testing time are excluded.

**Methods:** Total 158 test chemicals (94 liquids and 64 solids) were tested by the Vitrigel-EIT method and their judgments were compared with the GHS classification. According to the TG494, 89 test chemicals were predicted after excluding 12 acidic chemicals and 57 solids. In the revised test method, 107 test chemicals were predicted after excluding 12 acidic chemical preparations and 39 chemical preparations with the absolute differences over 0.1 between the A660 of test chemical preparation at 0 and 3 minutes after mixing.

**Results, Discussion:** The sensitivity, specificity and accuracy under the original applicability domain in TG494 were 95%, 67% and 80%, respectively. Meanwhile, those under the revised test method were 96%, 67% and 81%, respectively. These data demonstrated that the revised test method improved the number of applicable chemicals and also the predictability, concluding that the Vitrigel-EIT method can be used for not only liquids but also solids.

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## Development of guidance for IACUC members in Korea: Applying the 3Rs principles

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Applying the 3Rs (Replacement, Refinement, and Reduction) principles, as well as undertaking the ethical review of animal study protocols, have been legal requirements for Institutional Animal Care and Use Committees (IACUCs) in Korea since 2008. As of 2019, there were 410 IACUCs registered to the Animal Protection & Welfare Division of the Animal and Plant Quarantine Agency. Each IACUC is required to appoint at least one member that was recommended by a non-governmental animal welfare organization. Despite the importance of the 3Rs, limited information resources are available in Korean, especially for lay members. The Korean Animal and Plant Quarantine Agency announced a six-month research project with the aim of developing guidance for assisting IACUC members to carry out effective and efficient protocol review in line with the requirements of Korean laws and regulations. Two Korean Guides – “Guide for the Animal Study Protocols” and “Guide for the IACUC Lay Member” – were published in December 2020. The UK Laboratory Animal Science Association (LASA) and the Royal Society for the Prevention of Cruelty to Animals (RSPCA) permitted the use of their English-based resources – the “Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies” and “A Resource Book for Lay Members of Ethical Review and Similar Bodies Worldwide” – as the basis for the Korean versions. Our goal is to provide valuable resources for researchers, IACUC members and other relevant staffs to improve their administrative processes, and to support their roles in promoting both animal welfare and the quality of the science by applying the 3Rs principles. These two Guides could also help bridge the gap between scientists and animal welfare advocates by helping to improve ethical considerations and promoting a culture of care at all research institutions that use laboratory animals.



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## Development of a diet-induced model for non-alcoholic steatohepatitis (NASH) and fibrosis in a triple cell-type, spheroid-based liver-on-chip model with microfluidics

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Non-alcoholic fatty liver disease (NAFLD) is currently the most common form of chronic liver disease and may progress towards NASH associated with liver fibrosis, cirrhosis, and hepatocellular carcinoma. Despite ongoing efforts, there is no effective therapeutic treatment available for NAFLD-NASH. This is partly due to an incomplete understanding of disease mechanisms, absence of relevant biomarkers and predictive preclinical models for drug screening. We developed a disease-mimicking *in vitro* model which closely resembles the pathophysiology of liver fibrosis induced by lifestyle.

Primary human hepatocytes, Kupffer cells, and stellate cells were cultured in a matrix-free environment, resulting in formation of multiple uniformly sized spheroids. Fatty acids, carbohydrates, inflammatory and immunomodulatory factors were used at physiological concentrations to faithfully recapitulate disease development and progression of NAFLD-NASH. Development of steatosis was imaged by LipidTOX accumulation using confo-

cal microscopy and plate reader assays, and susceptible to treatment with the PPAR agonists fenofibrate or pioglitazone. Transcription and protein analyses confirmed expression of different collagen isoforms upon full disease induction.

In addition, we introduced microfluidic flow to our cell model to investigate the effect of homeostatic tissue perfusion versus conventional static culture conditions. A novel, customized liver-on-chip was developed in-house. The chip was 3D-printed using proprietary material that has very low drug adsorption to circumvent the shortcomings of the widely used polydimethylsiloxane (PDMS). Spheroids were subjected to continuous pump-driven flow for 2 weeks. Exposure to fatty acids and carbohydrates under flow conditions resulted in a more homogeneous distribution and size of lipid droplets, both per individual hepatocyte and per spheroid cross section, as compared to static culture conditions where droplet size was more variable.

We will further investigate the effect of microfluidic flow in our model on the therapeutic efficacy of reference compounds and compounds currently in clinical trials, using steatosis, inflammation, and deposition of collagen as read-outs.

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## A next generation risk assessment case study for coumarin in hypothetical cosmetic products

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Next Generation Risk Assessment (NGRA) is an exposure-led, hypothesis-driven approach that integrates New Approach Methodologies (NAMs) to assure safety without the use of animal testing. Over recent years several theoretical frameworks depicting a tiered and iterative approach to conducting a NGRA have been published (Berggren et al., 2017; Dent et al., 2018), although there is a lack of examples of implementation of these frameworks.

In this study we conducted a hypothetical safety assessment of 0.1% coumarin in a face cream and body lotion using only NAMs to inform a safety decision, focusing on the potential for systemic toxicity. Internal exposure estimates were generated using a physiologically based kinetic (PBK) model for dermally applied coumarin (Moxon et al., 2020). *In vitro* points of departure (PoDs) were generated from assays that investigated the po-



tential of coumarin to bind to pharmacologically active receptors (Eurofins Safety44 screen); cause immunomodulatory effects (BioMap Diversity 8 Panel); affect key cellular stress pathways (Hatherell et al., 2020) and high-throughput transcriptomics in multiple cell lines. *In silico* alerts for genotoxicity were followed up using the *in vitro* ToxTracker assay (Hendriks et al., 2016).

A risk assessment decision was made by comparing the generated *in vitro* PoDs to the estimated internal exposure (plasma C<sub>max</sub>) and calculating a margin of safety (MoS) distribution. The MoS (5<sup>th</sup> percentile) for both face cream and body lotion exposure scenarios were greater than 100. Coumarin can be concluded to be non-genotoxic, does not bind to any of the 44 targets and does not show immunomodulatory effects at consumer relevant exposures.

While this case study demonstrates the capability of using NAMs to make safety decisions about inclusion of coumarin in cosmetic products, confidence in the applicability of this approach to other chemicals and products will only come through sharing the experiences of other case studies.

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## Apoptosis and autophagy can be reduced with an iNOS-inhibitor in an oxidative stress retina organ culture model

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The overproduction of reactive oxygen species is defined as oxidative stress, which is associated with several retinal diseases, including glaucoma or retinal ischemia. H<sub>2</sub>O<sub>2</sub> can be used in *ex vivo* organ cultures to simulate oxidative stress, causing a strong neurodegeneration of the inner retinal layers (Hurst et al., 2017). Based on this model, novel therapeutic approaches can be evaluated. A potential neuroprotectant for this disease is the nitric oxide synthase (iNOS)-inhibitor 1400 W, which was evaluated here.

Porcine eyes from the local abattoir were obtained to avoid extra killing of animals. Oxidative stress was induced by adding 300 µM H<sub>2</sub>O<sub>2</sub> on day one for 3 h to the organ cultures. Simultaneously, 500 µM iNOS-inhibitor (1400 W, Merck Millipore) was added. Neuronal and glial cells were evaluated via qRT-PCR and immunohistology after four and eight days. In addition, Transmission electron microscopy (TEM) was performed. The following groups were compared: controls, H<sub>2</sub>O<sub>2</sub>, and H<sub>2</sub>O<sub>2</sub> + iNOS-inhibitor.

H<sub>2</sub>O<sub>2</sub>-induced retinal ganglion cell (RGC) loss was prevented with iNOS-inhibitor treatment. On mRNA level, a significantly increased expression of autophagic gene p62 due to H<sub>2</sub>O<sub>2</sub> was hindered by the iNOS-inhibitor treatment after four days. Likewise, a reduced expression of caspase 8 was seen after iNOS-inhibitor treatment (3.19-fold; p = 0.54) in comparison to the H<sub>2</sub>O<sub>2</sub>-stressed group (5.37-fold; p = 0.065). A late rescue of bipolar cells was noted in 1400W treated retinas. TEM revealed that cell morphology as well as the cell compartments of RGCs were protected from oxidative stress damage by the iNOS-inhibitor.

In conclusion, Strong degeneration in porcine retinas due to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress was prevented by treatment with the iNOS-inhibitor. Reduction of apoptosis resulted in cell rescue, in particular of RGCs. These results indicate, a potential neuroprotective role of the iNOS-inhibitor 1400 W in diseases affecting the inner retina. Moreover, this successful treatment further validates our oxidative stress porcine *ex vivo* model.

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## Development of an *in vitro* B cell assay for tetanus and diphtheria containing vaccine batch testing using antigen specific B cells from healthy donors

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**Background:** Protection against bacterial toxin-mediated diseases such as Tetanus, Diphtheria and Pertussis is mediated by antibodies. Induction of antibody production by toxoid vaccines containing inactivated toxins confers protection from these diseases. The aim of our project is, therefore, to develop a cell-based assay that mimics the induction of toxoid-specific antibodies *in vitro* in replacement for the currently required *in vivo* challenge and immunogenicity studies.

**Objectives:** We are developing an assay based on the stimulation of the B cell response to the vaccine antigen in tetanus/diphtheria toxoid-specific B cells sorted from human peripheral blood mononuclear cells isolated from buffy coats. The protocol is designed to elicit a recall response to tetanus toxoid (TT) and diphtheria toxoid (DT).

**Methods:** Antigen-specific memory B cells are sorted from buffy coats, stimulated with TT/DT and co-stimulated with low concentrations of CpG ODN. B cell responses is quantified via antibody production in antigen-specific IgG ELISpot.

**Results:** Exposure of TT/DT-specific memory B cells to vaccine antigen induces differentiation into antibody-secreting cells and their specificity is confirmed by the detection of specific IgG antibodies in ELISpot. The results obtained indicate that the established protocol with TT/DT specific B cells from buffy coats delivers a specific response to the antigen and can be used to evaluate the immunogenicity of TT and DT vaccine compounds. Identification, isolation and quantification of TT/DT specific IgG memory B cells in this assay allow reduced variability of a cell-based assay and improves standardization for routine use in a future.

**Conclusions:** Our test system will serve to prove the functional integrity of the vaccine bulk antigens, by confirming the induction of the expected specific human antibody response. It might offer a potential alternative to time-consuming animal experiments currently used for batch testing of vaccines containing TT and DT components.

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## Improving animal research: Enhancing systematic review methodology

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Systematic Reviews (SR) are increasingly important for implementing the 3R measures (de Vries et al., 2014). E.g., they are used to validate replacement methods (Replace), preventing unnecessary experiments (Reduce) or assessing study quality (Refine). However, SRs of animal studies are still relatively uncommon, and methods not always evidence based. We aim to make SRs more robust and easier to perform. Here we present three projects that will help to improve the methodology.

In the first project we focus on searching for animal studies. With SYRCLE we are co-developing animal search filters for Embase.com, PsycInfo, and Web of Science. These filters will increase specific retrieval of animal studies, decreasing the number of references needed to screen without missing relevant references.

In the second project we compared different software tools for reference screening for in- or exclusion. Screening is one of the most time-consuming parts of an SR. There are many tools available, but it is not always clear how these tools differ. We performed a feature analysis where we assessed which tools offered which features relevant for screening. The results make it easier for researchers to select the tool appropriate for their SR (Van der Mierden et al., 2019).

In the third project we analyze how data extraction from graphs influences the SR analysis. Ideally, the values of outcomes of interest are presented in the text, but frequently they are only presented in graphs. First, we compare variability between researchers extracting the graphical data, and afterwards we aim to analyse the potential effect of the differences between the extracted and original data on the analysis by simulation studies, this project is currently ongoing

Improving and facilitating SR methodology will lead to a lower threshold for performing SRs and higher quality SRs. These SRs in turn help to better implement the 3Rs.

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## A digital tool based on transcriptomic data for the integration of biological fingerprint analogies in the read-across approach

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Since March 2013 the use of animal testing for cosmetic ingredients has been banned in the EU. To assess systemic toxicity of new cosmetic ingredients, read-across approaches based on structural analogy are one of the next generation risk assessment approaches currently used. However, structural analogies are not sufficient to ensure similar toxicological behavior and are limited to ingredients with a defined structure.

Advances in omics technologies have allowed the emergence of public databases containing full genome transcriptomic profiles of thousands of compounds at many different biological conditions (concentration, time and biological system). By comparing transcriptomic fingerprints, such databases can support read-across approaches based on biological activity analogy.

In this context, a digital tool integrating transcriptomic profiles from Drug Matrix, Open Tg-Gates (Igarashi et al., 2015) and Connectivity Map was developed, allowing 1) to assess similarity between compounds and 2) to investigate the main biological pathways targeted by each compound. Using Hallmark gene sets (MSigDB), Gene set enrichment analysis (GSEA, Subramanian et al) was applied to calculate Normalized Expression Scores. Pairwise similarity scores were then computed from the GSEA results using Pearson's correlation. In addition, wind-rose plots of implicated pathways are constructed to help characterize the mechanisms of actions (MOAs) and allow chemicals grouping based on their transcriptomic fingerprints.

High biological similarity scores were observed for compounds with similar structure and for compounds that were not previously identified as analogs by conventional read-across approaches. This tool could be useful to enhance structural based read across approaches and to assess analogy between chemicals with no defined structure.

Next steps will consist of 1) further characterizing observed differences/similarities between compounds towards a better understanding of compound's MoAs and 2) developing and evaluating a classifier including results obtained with this digital scoring tool

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## Primary or iPSC-derived cell-based cytotoxicity assays to assess potential safety risks of engineered T cell therapies *in vitro*

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Engineered T cell therapies, such as Chimeric Antigen Receptor (CAR) and T Cell Receptor (TCR) T cells play a major role in the field of immuno-oncology and offer great promise in becoming highly specific therapies against solid tumors. Solid tumors generally lack the expression of tumor specific target antigens, posing a significant safety challenge. This can potentially result in life-threatening side effects, as such, assessing the safety of engineered T cell therapies is a critical step during early development and before filing for Investigational New Drug (IND) status.

We have generated an *in vitro* safety profile for CAR-T cells targeting the "Human Epidermal growth factor Receptor 2" (HER2). This receptor is found to be overexpressed in 20-30% of invasive breast carcinomas and ovarian cancers. To determine which tissues are most at risk for unwanted reactivity by the CAR-T cells, *in silico* analysis for expression of HER2 was performed. Subsequently, HER2 protein expression in various tissues was validated by staining cells with a HER2 antibody and determining percentage positive cells by flow cytometry. Primary tissues and iPSC-derived cell with high and low HER2 protein expression were selected, characterized and utilized for *in vitro* co-culture assays to evaluate on-target off-tumor and/or off-target cytotoxicity of our HER2-CAR-T. Readouts for the *in vitro* cytotoxicity assays included measuring target cell viability by flow cytometry and/or HCA and T cell activation by cytokine release.

Our study generated high quality data that provided insight into the safety of the HER2 targeting CAR-T cells. Moreover, we were able to demonstrate the value of using iPSC-derived cells in de-risking selected tissues against unwanted reactivity of engineered T cell therapies. Our strategy to generate a safety profile *in vitro* for T cell therapies is robust can be applied during both early and late-stage testing of the therapeutic product.

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## Generation of novel, integrated and internationally harmonized approaches for testing metabolism disrupting compounds (GOLIATH)

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The GOLIATH project focusses on one of the most urgent regulatory needs in EDC testing, namely the lack of methods for testing EDCs that disrupt metabolism – chemicals collectively referred to as “metabolism disrupting compounds” (MDCs). MDCs are natural and anthropogenic chemicals that have the ability to promote metabolic changes that can ultimately result in obesity, diabetes and/or fatty liver in humans. GOLIATH will generate the world’s first integrated approach to testing and assessment (IATA) specifically tailored to MDCs. With a focus on the main cellular targets of metabolic disruption – hepatocytes, pancreatic endocrine cells, myocytes and adipocytes – GOLIATH will develop new and optimize existing methods that span the entire adverse outcome pathway (AOP) spectrum. GOLIATH will provide key information by which mode of action MDCs disrupt pathways by incorporating multi-omics and translating results from *in vitro* and *in vivo* assays to adverse metabolic health outcomes in humans at real life exposures. Given the importance of international acceptance of the developed

test methods for regulatory use, GOLIATH will link with ongoing initiatives of the OECD for test method (pre-)validation, IATA and AOP development. With a consortium comprised of world-leading experts, GOLIATH will be pivotal in the development of an internationally harmonized strategy for testing MDCs, with the ultimate aim of slowing the worldwide rise in metabolic disorders that have reached “Goliathan” proportions.

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## SMAFIRA: Smart feature based interactive ranking to retrieve possible alternatives from the literature

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Any researcher who plans to undertake a scientific project relying on animal experimentation in one of the Member States of the European Union has to apply for project authorization (Article 37, Directive 2010/63/EU). The application includes, amongst other things, 1.) the relevance and justification of the use of animals, 2.) the application of methods to “replace, reduce and refine” the use of animals in procedures (3R principle), and 3.) the avoidance of unjustified duplication of procedures. The information needs to be accompanied by an evaluation of the current scientific knowledge. PubMed/MEDLINE is a prominent resource to search for present state of biomedical knowledge and researchers routinely use it (and its provided search tools) to fulfill the legal requirements. We here introduce a project that aims to support a 3R relevant information retrieval from PubMed/MEDLINE. The SMAFIRA (smart feature based interactive ranking) project strives to elaborate a tool that ranks abstracts according to their equivalence to a given reference document in terms of scientific objectives. In addition, it shall allow for interactive filtering of searched-for methodological approaches (*in vivo* vs. *ex vivo* vs. *in vitro*). Ranking and filtering approaches are fueled by up-to-date machine learning algorithms. With this tool we want to support scientists in their search for alternatives to animal testing based on the experimental purposes.

**Presentation:** Poster



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## Animal models used for regenerative medicine research in Costa Rica. A case of study: Skin wound healing

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In the last two decades, a series of projects aiming towards the establishment of regenerative medicine technologies for skin injuries in Costa Rica have been developed. Joint efforts have been made between different state hospitals and universities, among which the Costa Rican Institute of Technology stands out as a protagonist.

Some of those projects have been limited to animal-cell culture studies, while others have required the use of animal models to verify the potential of cell therapies, in pre-clinical assays. This research has allowed the establishment of protocols designed to evaluate the wound regeneration rate and quality in various murine models, which in turn has allowed their refinement. To evaluate the regeneration potential of Adipose-derived Stromal Cells (ASC) seeded in a biological scaffold, this skin injury murine model was used.

One squared centimeter acute full-thickness skin wound was produced in the interscapular area of adult male Balb-C mice, and four different treatments were applied as follows: a) ASC in saline solution, B) ASC seeded in a biological scaffold (agarose), C) agarose without cells, D) a commercial agent for wound healing as a positive control and D) saline solution as a negative control. The animals were assessed, and the injuries measured on a daily basis until the wound was completely closed. After that, samples were taken, and a histological evaluation was performed.

Preliminary tests have shown that ASC seeded in an agarose scaffold tend to reduce the wound faster during the first week compared to ASC in saline solution, although complete closure was obtained around day 12 in both treatments. Other treatments showed lower regeneration rates compared to ASC treatments.

These experiments constitute some of steps taken in Costa Rica towards the use of cellular therapies at a clinical level.

**Presentation:** Poster

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## Establishment of a risk assessment method and threshold of toxicological concern (TTC) concept for skin sensitization by non-animal approaches

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**Introduction:** Recently, several *in vitro* skin sensitization tests have been listed in the OECD guidelines. The threshold of toxicological concern (TTC) is a threshold value for human exposure that does not show any obvious adverse effects at the lower doses for all chemicals. In this study, we aimed to develop a quantitative prediction model using *in vitro/in silico* data and establish a TTC concept for skin sensitization.

**Materials and Methods:** The EC3 value, which is the endpoint of the LLNA, was used as the objective variable, and data on 120 substances were extracted from the dataset published by Cosmetics Europe. *In vitro* tests (DPRA, KeratinoSens and h-CLAT) data and physico-chemical properties were used as the explanatory variables. A quantitative prediction model for EC3 was developed using support vector regression (SVR), which is a machine learning approach. Predicted EC3 values were used to establish a no expected sensitization induction level (NESIL), and acceptable exposure level (AEL) for each chemical was calculated by dividing NESIL by sensitization assessment factor (SAF). Then by fitting gamma distribution of the AELs using a negative log (10) scale, 95 percentile probability was calculated as the Dermal Sensitization Threshold (DST). Finally, conversion of the DST to the concentration in a face cream was done as an example of the application of this concept.

**Results and Discussion:** This prediction model was validated by 3-fold cross validation, and the accuracy of prediction of potency class in four categories was 45.8%. Assuming 20% of all chemicals to be skin sensitizers, the exposure threshold (micro g/cm<sup>2</sup>) for women's face cream was 3.99 (95 percentile). Furthermore, the concentration threshold of this type of products was calculated as 0.26%. This TTC concept will be useful for the safety evaluation of impurities in cosmetic ingredients as a non-animal approach.

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**Presentation:** Poster



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## Current status of the RE-Place database comprising expertise on the use of NAMs in Belgium

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In the life sciences, new and innovative technologies are continuously evolving. Techniques such as computer modelling, artificial intelligence, sophisticated cell cultures, and organ-on-a-chip have seen important advances since their initial development. When these methods contribute to the overall replacement and reduction of animal testing, they are referred to as “New Approach Methodologies (NAMs)”. NAMs also cover more “conventional” methods that aim to improve the understanding of toxic or biologic effects. Due to the fast progress of these technologies, (young) scientists can struggle to find relevant information on NAMs. In order to facilitate their search, the Flemish government initiated the project “RE-Place” in 2017 which aims to collect the existing expertise on NAMs in one central database. The Brussels Capital and Walloon regions later joined this project, making it a national initiative. RE-Place is coordinated by Sciensano and the Vrije Universiteit Brussel and consists of three phases: (1) an exploratory survey to identify experts using NAMs, (2) the development of an online tool to collect specific information on these methods, and (3) the compilation of an easy-to-use database, integrating all acquired data. In March 2021, the database contained over 150 NAMs covering a wide array of scientific areas. This open access database, available via [www.RE-Place.be](http://www.RE-Place.be), provides different stakeholders (regulators, industry, academia, government) reliable information on NAMs, while linking it to a direct contact. Furthermore, it enables scientists to (i) promote their work, (ii) exchange experiences, and (iii) engage in new collaborations by connecting them with their peers. By facilitating access to the available expertise and improving communication, RE-Place will grow as a powerful tool to stimulate the use of NAMs, promote their (further) development, and allow the identification of knowledge gaps to better allocate future funding. Overall, RE-Place will contribute to the replacement and reduction of animal testing, wherever scientifically possible.

**Presentation:** Poster

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## Telemetry as method to assess severity in sheep

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Ensuring animal welfare and assessing severity are fundamental issues that scientists undertake whenever animal experimentation is needed. Currently, clinical scoring is the gold standard to assess procedural severity in sheep. However, sheep are prey animals and welfare concerns may be difficult to assess by an observer physically present and close to the animal. We present here the results of contactless monitoring of heart rate (HR), HR variability, general activity and body temperature via telemetry after surgery and their comparison to clinical scoring. An M01 telemetric device (DSI, Harvard Bioscience, Inc.) was implanted subcutaneously in the right neck of four German blackheaded mutton ewes (4-5 years, 77-115 kg). Four weeks after implantation, the sheep underwent tendon ablation of the left M. infraspinatus at the greater tuberosity of the proximal humerus. After both surgeries, values for HR, HR variability, general activity and temperature were recorded by the M01 and clinical scoring was performed. Analysis of telemetric data after both surgeries revealed slightly elevated HR and reduced general activity on the first postsurgical day compared to baseline, which were not found in clinical examinations. Furthermore, the sheep showed decreased temperatures up to day five after transmitter implantation. Interestingly, no correlation between clinical scoring and telemetric data could be determined. In order to classify the data with regard to severity assessment, a Support Vector Machine on the basis of k-means class-labeled telemetric data was trained. The application of the model highlighted the impact of both surgical interventions on individual sheep. Our results indicate that severity assessment of surgical interventions in sheep can be measured more effectively via telemetry than clinical scoring. Furthermore, classification of telemetric data facilitates and enhances quantitative analysis of different procedures in sheep. Consequently, telemetry offers a valid method for the refinement of animal experimentation in sheep.

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**Presentation:** Poster



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## New approach for systemic toxicity hazard assessment based on alternative methods to animal testing in support of safety decision-making

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To ensure the safety of new ingredients when used in cosmetic products, development of New Alternative Methods (NAMs) addressing Acute Oral Toxicity (AOT) and Repeated-Dose Toxicity (RDT) has become essential since the animal testing ban. In this context, we propose a new approach that could feed the Next Generation Risk Assessment including *in silico* approaches as well as *in vitro* models to support decision-making. For AOT, we developed an *in silico* profiler and a multiparametric approach for the oral LD50 prediction. RDT assessment was evaluated using a panel of traditional and emerging technologies from transcriptomic analysis to tissue specific models. The approach was assessed on a set of compounds without *in vivo* AOT alerts including, well-known chemicals (Amiodarone, Acetaminophen), a negative control (Mannitol) and one hair dye with an unfavorable SCCS' opinion due to genotoxicity alerts. It is also known to cause *in vivo* liver and heart histopathological alterations in rodents after oral repeated dosing from legacy studies.

The LD50 prediction correctly identified non acute toxic compounds (3/4). No toxicity was observed for Mannitol whereas Amiodarone and Acetaminophen showed *in vitro* mitochondrial alterations described as one of their mechanism of action involved in the chemical-induced hepatotoxicity *in vivo*. Finally, a strong decrease in albumin secretion in the 3D-liver spheroid assay, and a decrease of cardiomyocytes' conduction velocity and contraction forces were observed *in vitro* for the hair dye.

This new approach provided relevant elements contributing to hazard characterization for the 4 test articles, which must be assessed on a larger set of compounds to better evaluate the value of NAMs in the assessment of systemic toxicity.

Next steps will consist of 1) enriching the approach for reproductive toxicants assessment, and 2) integrating the kinetics to increase the relevance of the data in realistic use conditions.

**Presentation:** Poster

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## Assessing reproducibility, robustness and predictivity of an *in vitro* method to assess DIO1 inhibition in human liver microsomes

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Impairment of thyroid hormone homeostasis has been associated with several adverse effects. Regulatory requirements are increasing to identify different mode of actions (MoA) impacting thyroid hormone (TH) signaling pathways. Therefore, robust and predictive *in vitro* methods for the identification of substances affecting the TH system are needed. The European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) is coordinating the validation of multiple *in vitro* methods focusing on different thyroid MoA by cooperating with a network of EU laboratories (EU-NETVAL).

Deiodinases (DIO) are local regulators of TH action by enzymatically activating or inactivating TH via deiodination. DIO1, one of the three isoforms, serves as one main source for circulating T3 via deiodination of T4 in the thyroid and plays a role in recycling of iodide via deiodination of inactive TH metabolites.

Jointly with the method developer, a non-radioactive approach to determine substance-induced DIO1 inhibition based on iodide release in human liver microsomes was transferred to our laboratory and established following the GIVIMP concept (OECD, 2018). The released iodide was quantified via colorimetric change in the Sandell-Kolthoff reaction.

The relevance assessment (Part 1) consisting of six different DIO1 inhibitors in five independent runs could show robust and consistent dose-dependent decrease of iodide release activity in human liver microsomes for all tested inhibitors (e.g., IC50 values for the known DIO1 inhibitors: 6-Propyl-2-thiouracil = 3.8 µM and Aurothioglucose = 0.5 µM). Acceptance criteria were derived for an intra-lab validation study (Part 2) to assess predictivity with ≥ 30 blinded substances, selected by an international thyroid expert group.

The DIO1 inhibition assay using human liver microsomes is a promising *in vitro* assay to assess potential DIO1 inhibition of chemicals. Robustness and reproducibility could already be shown in Part 1. The predictivity assessment (Part 2) is ongoing and data are expected in Q2/2021.



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**Presentation:** Poster

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## Miniaturizing of liver metabolomics *in vitro* – A new screening approach to generate metabolic fingerprint in HepG2 cells

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Metabolomics *in vitro* (MIV) enables to predict the mode of action of liver toxicants in HepG2 cells. The currently used method at BASF (LUMOX-MIV) is robust, reproducible and predictive for different mode of actions (Ramirez et al., 2018). LUMOX-MIV is appropriate for testing chemicals in the regulatory context but is not applicable for a fast and low-cost screening approach. The aim of this study was to miniaturize the method on a 96-well plate ( $\mu$ MIV) and compare the data with LUMOX-MIV. As a proof of concept, bezafibrate, a well-established quality control, and seven additional substances known to cause liver toxicity through different modes of action (aroclor, pendimethalin, b-naphthoflavone, WY-14643, acifluorfen, fluoroglycofen-ethyl and ketoconazole) were tested with both methods.

After testing 7 different endpoints, the optimal cytotoxicity assay for dose selection was defined as a multiplexing approach combining ATP measurement with membrane integrity assessment. For the substance testing, HepG2 cells were cultivated on Lumox dishes (35mm, Sarstedt) by seeding 200 000 cells/ dish for LUMOX-MIV, and 15 000 cells/well in a 96 well plate for  $\mu$ MIV. After 24 hours, each substance was added. 48 hours later, the assay was stopped by harvesting the foil of the lumox dishes, freezing, and quenching it with DCM/EtOH (LUMOX-MIV). For  $\mu$ MIV, Isopropanol 80% was added to the wells, and the plates were frozen subsequently. The foils/plates were extracted and analyzed by LC-MS/MS for metabolic profiling.

Bezafibrate treatment showed a clear metabolic change compared to vehicle treated cells. In both systems consistently, the

lipid and energy metabolism were particularly altered, in line with the *in vivo* MoA of bezafibrate which acts as a lipid-lowering agent. The metabolome analysis for the other 7 substances is ongoing and will be presented. The first results postulate that the  $\mu$ MIV method is a promising *in vitro* tool for screening approach in toxicology and pharmacology.

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**Presentation:** Poster

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## Activities contributing to the deletion of the animal test for irreversibility of tetanus toxoids

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Tetanus vaccines are prepared from chemically inactivated tetanus neurotoxin (TeNT). This detoxified material is called tetanus toxoid. Safety tests are prescribed for each toxoid batch to make sure that it has been sufficiently detoxified. According to the European Pharmacopoeia, these safety tests have to be performed as toxicity tests in guinea pigs. One of these tests that was prescribed until recently addressed the irreversibility of the inactivation: In order to demonstrate that there is no “reversion to toxicity” during storage, the toxoid had to be stored at 37°C for six weeks before being tested in guinea pigs. We have investigated the relevance of this irreversibility test:

Comprehensive literature research and communications with vaccine manufacturers revealed no convincing evidence that any reversion events have ever been observed during the production of tetanus vaccines at all.

Moreover, using the binding and cleavage (BINACLE) assay as an *in vitro* method for the detection of active TeNT (Behrendorf-Nicol et al., 2013), we could show that the toxin rapidly loses its activity at 37°C. Consequently, active TeNT molecules that may potentially arise in a tetanus toxoid owing to reversion events will no longer be detectable after the six-week storage period, anyhow.

Based on these findings, we concluded that the prescribed test for irreversibility has no relevance for the safety of tetanus vaccines (Behrendorf-Nicol and Krämer, 2019). We presented our findings to the Expert Groups of the European Pharmacopoeia Commission to stimulate discussions on the possible deletion of this animal test. These discussions have ultimately led to the de-



letion of the test for “irreversibility of toxoid” from the Pharmacopoeia monographs for tetanus vaccines for human and veterinary use.

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**Presentation:** Poster

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## Deiodinase 1 in human liver microsomes is inhibited by organic and inorganic gold compounds and gold nanoparticles

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The hypothalamus-pituitary-thyroid axis (HPTA) plays a crucial role in energy homeostasis of adults and normal fetal brain development. Disruption of the HPTA has been linked to impaired neurodevelopment of the offspring and might be mediated by thyroid disrupting compounds.

Deiodinase 1 (DIO1), is a seleno-cysteine-containing enzyme in the HPTA which activates thyroid hormone via production of the biologically active thyroid hormone T3 in the thyroid and deactivation of thyroid hormone metabolites via deiodination in excreting organs like the liver or kidney.

Several inhibitors of DIO are known including some gold-containing pharmaceuticals. The aim of the study was to investigate gold-induced deiodinase inhibition of different gold-containing compounds including organic substances, inorganic salts, and gold nanoparticles (AuNPs) in the DIO1-Sandell-Kolthoff (DIO1-SK) assay.

The DIO1-SK assay uses human liver microsomes as a source for DIO1 to detect substance-induced inhibition of iodide release from iodothyronine. The Sandell-Kolthoff reaction is used to quantify the released iodide by colorimetry via reduction of yellow  $Ce^{4+}$  to colorless  $Ce^{3+}$  with a catalyzing function of iodide.

Aurothioglucose, sodium aurothiomalate and auranofin, three gold containing organic compounds that were used to treat rheumatoid arthritis, showed dose-dependent iodide release inhibi-

tion with similar IC50s in the range of 0.49 to 0.75  $\mu M$ . Their structural analogues lacking the gold cation did not lead to treatment-related iodide release inhibition. Two gold salts, Gold(I) and Gold(III) chloride showed comparable iodide release inhibition as the organic gold compounds tested. Further, testing 5 nm AuNPs in the DIO1-SK assay inhibited iodide release activity whereas larger AuNPs showed no effect on DIO1 activity.

We could show the susceptibility of DIO1 to inhibition by different classes of gold-containing substances and, potentially, AuNPs based on their size. The DIO1-SK assay might be a valuable *in vitro* method for the detection of DIO disrupting substances.

**Presentation:** Poster

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## The threshold of toxicological concern (TTC) is a pragmatic risk assessment tool for the safety assessment of cosmetic ingredients with low consumer exposure

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The Threshold of Toxicological Concern (TTC) is a pragmatic and conservative tool for the risk assessment of substances. It is based on the principle of establishing a human exposure threshold value for all chemicals, below which there is a very low probability of a possible risk to human health. Such threshold values may be identified for many substances on the basis of their chemical structures and the known toxicity of chemicals sharing similar structural characteristics. The TTC concept has been internationally accepted and used in a wide range of regulatory contexts. The human exposure threshold values have been originally derived from oral toxicity data on cancer and non-cancer toxicity endpoints (Munro et al., 1996). This database has been substantially enlarged by the COSMOS database, an enhanced oral non-cancer TTC dataset on a larger chemical domain, thereby resulting in a new, transparent and public TTC database which also includes 552 cosmetics-related chemicals (Yang et al., 2017). The 5<sup>th</sup> percentile point of departure value for each Cramer Class was determined, from which human exposure TTC values have been derived. The COSMOS-plus-Munro federated dataset provided TTC values of 46, 6.2 and 2.3  $\mu g/kg$  bw/day for the Cramer Classes I, II and III, respectively. Overall, the TTC is accepted by regulatory authorities and most scientific committees, and there is broad application potential for use in safety assessments of cosmetic ingredients. Cosmetics Europe has prepared several



case studies which demonstrate that the TTC approach is a sufficiently conservative approach to safeguard the consumer. Overall, the TTC concept is useful to avoid animal testing and successfully evaluates the safety of cosmetic ingredients for which the consumer exposure is low.

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**Presentation:** Poster

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### A new growth factor-free human cell-based *in vitro* angiogenesis assay for testing angiogenesis inhibitors

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Inhibition of angiogenesis, de novo formation of blood vessels, is a relevant mode of action which, may lead to malformations. *In vitro* angiogenesis assays are often complex and require the addition of growth factors (GF) (Toimelaa et al., 2017). We developed a human based *in vitro* angiogenesis assay without external GF to screen anti-angiogenic effects of chemicals.

A 96well based assay was developed by co-culturing human fibroblasts (CI-huFIB, Inscreenex) at Day 0 with addition of primary HUVECs (PromoCell) at Day 3. At Day 4 and 8 cells were treated with test items with known antiangiogenic/teratogenic effects (Digoxin, Levamisole, 2-Methoxyestradiol), two teratogenic substances without angiogenic inhibiting effects (Phenytoin, Methimazole) and one without anti-angiogenic and teratogenic effects (D-Mannitol) (Toimelaa et al., 2017). On day 14, the HUVECs were stained with rabbit- $\alpha$ -Factor VIII related antigen (Zytomed) to quantify the HUVEC tube formation by measurement the network length and branching points (IncuCyte, Sartorius). MTT viability test was performed in parallel to exclude general cytotoxic effects.

No external GF was needed to build a reproducible tubular network proven by Digoxin in > 15 runs. Levamisole, Digoxin, Phenytoin and 2-Methoxyestradiol showed lower IC50 for the network length and branching points than cytotoxicity indicating a selective anti-angiogenic effect, while Methimazole and D-Mannitol neither showed any inhibitory effect for inhibition of tube formation nor cytotoxicity up to 1000  $\mu$ M in line with Toimelaa et al. (2017). Phenytoin showed anti-angiogen-

ic properties in our assay but was inactive in the assay of Toimelaa et al. (2017). Phenytoin is often considered as wound healing inducer, but Eser et al. (2021) could demonstrate anti-angiogenic effects in mice.

This GF-free human cell-based *in vitro* angiogenesis assay is robust, reproducible and predictive. Further testing is needed but it could be a useful tool to investigate angiogenesis in toxicology and pharmacology in the future.

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**Presentation:** Poster

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### The BINACLE (binding and cleavage) assay for measuring the activity of botulinum neurotoxin *in vitro*

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Botulinum neurotoxins (BoNTs) are highly potent bacterial toxins inducing a flaccid paralysis. The muscle-relaxing effects of the serotypes BoNT/A and BoNT/B are exploited in clinical and aesthetic medicine to treat a broad spectrum of disorders associated with muscle overactivity. In order to avoid toxic side effects, the potency of each BoNT batch has to be precisely determined. The "gold standard" method for this purpose is a test measuring the lethal toxin dose (LD50) in mice, which causes severe distress for the test animals. Although some approved alternative methods exist, none of them is applicable to all relevant BoNT products and freely available for all potential users.

We have developed a method for measuring the activity of BoNT/A and BoNT/B *in vitro* based on the two most important specific characteristics of these toxins, namely their receptor-binding and proteolytic properties (Wild et al., 2016; Behrendorf-Nicol et al., 2018). In-house characterization studies demonstrated that this BoNT BINACLE (binding and cleavage) assay is highly sensitive and applicable to all approved BoNT products, thus meeting the basic prerequisites to serve as an alternative to the LD50-based animal tests. It was further shown that the method can be straightforwardly transferred to other laboratories.



Currently, an international collaborative study is ongoing to promote acceptance of the BINACLE assay by BoNT manufacturers and regulatory authorities. In future, the BINACLE assay could allow animal-free activity determinations of BoNT products and could thus lead to a noticeable reduction in animal numbers.

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## Mining retinoic acid pathway related biomarkers of vertebrate developmental toxicity in the zebrafish embryo model

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The assessment of potential developmental toxicity is a critical component included in the data package for chemical safety. As *in-vivo* studies involve the use of many animals, there is, consequently, a need for an alternative protocol to rapidly identify developmental toxicity properties for young candidate molecules. The zebrafish embryo model is increasingly used in the field of toxicology. It offers the opportunity to study the development of an intact vertebrate embryo in an animal-free regulatory context. The fast-developing egg allows for a cheaper and less time-consuming whole vertebrate organism study, with relevance for human when studying highly conserved developmental mechanisms. The function of the retinoic acid (RA) pathway is highly conserved in vertebrate embryogenesis. The concentration of its principal actor all-trans-retinoic acid (ATRA) is strictly maintained locally by a balance between synthesizing enzymes

such as *aldh1a2* and metabolizing enzymes of the *cyp26* family. This balance creates local gradients essential for vertebrate embryo development. ATRA interacts with morphogenetic regulators (MR) such as *fgf8*, *wnt* and *shh*, driving complex morphogenetic processes like neural tube patterning. Our objective was to understand how changes in the RA pathway transcriptome relate to malformation. We studied transcriptomic and morphological data of zebrafish embryos exposed to ATRA (7.5 nM) for different durations (2 h, 4 h, 6 h, 24 h, 48 h, 72 h, 120 h). The increased duration of exposure leads to increase consequences in the morphological readout. In the transcriptome read out the stronger level of relative gene expression changes appears in the first duration exposure, specifically in the RA metabolism (*cyp26a*), in *hox* genes (*hox2*), and other MO (*fgf8b*), highlighting the relevance of the RA pathway in the study of developmental toxicity in the zebrafish embryo. It allows us to select RA pathway-related genes as biomarkers for developmental toxicity, which we anticipate will be responsive to a wide array of teratogens.

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## OECD approval is not the end of the story – Are existing test methods (OECD 442C, D and E) applicable to nanomaterials?

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Since January 2020, Commission Regulation (EU)2018/1881 is introducing REACH registration requirements regarding nanomaterials (NM). This requires characterization of nanoforms, performance of chemical safety assessment including qualification of adaptation of test methods to nanoforms and modification of information requirement where the test method is not applicable. For chemicals, a battery of three *in vitro* methods to test for skin sensitization potential was validated and approved by OECD (DPRA, LuSens, h-CLAT, the OECD TG 442C, D and E, respectively). In the BMBF-funded project Aerosafe (031L0128C), the applicability of these methods to NM was evaluated.

Nine different NM (i.a. CeO<sub>2</sub>, BaSO<sub>4</sub>) were tested in three assays to predict skin sensitization based on the adverse outcome pathway (i) protein interaction (DPRA), (ii) activation of keratinocytes (LuSens) and (iii) activation of dendritic cells (h-CLAT). Modifications of assay protocols were introduced: characterization of NM and homogeneous and reproducible NM suspension by using cuphorn sonication.



h-CLAT and LuSens assays include MTT cytotoxicity testing. ATP-cytotoxicity assays were performed in addition if interference of the NM with the MTT assay were apparent. The h-CLAT assay uses flow cytometry which is sensitive to NM particles. "Density gradient centrifugation" was introduced to separate the NM from the cells before applying to the flow cytometer. The DPRA protocol calls for test items in aqueous solutions, whereas insoluble NM form suspensions. According to the prediction model of the DPRA, the results with NM are regarded as "inconclusive" rather than "negative". Obviously, the prediction model of the DPRA needs to be amended for insoluble test items.

The well-established assays on skin sensitization are applicable to NM with adaptations of the test item preparation, assay protocols and prediction models. Further tests are needed to learn more about the applicability of the OECD442C, D and E test battery for a wider range of NM.

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## Sensitization potential of medical devices detected by methods *in vitro* and *in vivo*

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Medical devices have to be tested before marketing in accordance with ISO EN 10993-10 in order to avoid skin sensitization. The objective of this pilot study was to evaluate sensitization potential of selected medical devices combining *in vitro* and *in vivo* methods. DPRA (OECD TG 442C) is an *in chemico* procedure addressing the molecular initiating event leading to skin sensitization, namely protein reactivity, by quantifying the reactivity of test chemicals towards model synthetic peptides. LuSens (OECD TG 442D) is an *in vitro* procedure based on human keratinocytes transfected with stable reporter gene for luciferase. The measured luciferase signal reflects activation of the relevant signaling pathway by sensitizers. Local Lymph Node Assay:DA (OECD TG 442A) is a non-radioactive method using mice, measuring the proliferation of lymphocytes induced in the auricular lymph nodes after application of the test material (extract). So far, 39 samples of medical devices have been tested, 6 were positive in LLNA, 9 positive in DPRA and 8 positive

in LuSens (depending on extract vehicle). Regarding positive classification, better concordance was found between DPRA and LuSens (4/9 positive DPRA results), while LLNA provided three positive classifications concordant with both LuSens and DPRA. This study demonstrated good agreement between *in vitro* and *in vivo* method results regarding the absence of skin sensitization potential, however, discrepancies in positive classifications were recorded. The mismatch between *in vitro* and *in vivo* results might be caused by specific response of the immune system of the living organism. The *in vitro* methods require optimization of procedure, in particular the choice of appropriate extraction vehicle, sterility of the extracts and applied concentrations/volumes.

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## Identifying fish – Methods for tagging and marking

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In many areas of fish research, it is necessary to identify individuals or groups. Sometimes, this can be achieved by using the fish's natural pattern and other unique markings, but often the fish must be marked or tagged. There are a number of documents describing one or more methods, but an easy-to-use guide that includes both fish welfare and user perspective was lacking. In this project we therefore summarized and evaluated all the most common methods for marking/tagging fish. The best choice of method depends on the reason for marking/tagging, fish species, habitat and size of the animal. Also, whether the animals should be possible to identify on a distance or not influence what choice to make. If the reading will be done on a distance or movement data or position is needed, the choice stands between using microchips (e.g., PIT-tag) or telemetry/biologgers. Genetic identification gives more detailed data about inbreeding and population structure than other methods and have low impact on the fish but cannot be read at a distance. Coded-wire tags, VI Alpha (visible implant alphanumeric) are easy methods to use that have little negative effect on the



fish and they can be used for separating individuals, while VIE (visible plant elastomer) is an equivalent for separating mainly groups. There are methods to create patterns in the otoliths that can be a suitable method for group marking. Although removing pelvic fins, removing maxilla (part of the mouth), and spray marking are legal in Sweden, we discourage the use of these methods due to animal welfare reasons. We believe our profiling of the methods and the complimentary decision tree will make it easier to choose the most suitable method for marking, tagging and identification, thereby reducing the number of fish used and the negative impact on the fish.

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## The problem of pain in animal experimentation

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Untreated or mistreated pain in animals in laboratories presents numerous scientific and ethical concerns in research. In North America, Europe, and Asia, various animal welfare laws and guidelines exist to minimize pain in animals used in experiments, yet these animals are often given inadequate pain relief and critical information about pain management is frequently underreported in scientific publications. Traditional tools to assess pain in animals are inadequate, and more modern tools are infrequently implemented. Additionally, mismanaged pain relief and monitoring comprise a concerning percentage of welfare violations in laboratories in the United States. Collectively, this results in an alarming but underreported number of animals suffering with pain in laboratories. This is not only ethically unacceptable, but also introduces potentially catastrophic confounds into experimental data; animals experiencing pain may exhibit reduced food and water intake, impaired sleep, reduced motor activity, slower wound healing, and increased stress-related hormone production.

We will present current data on the number of pain-related animal welfare violations in top U.S. laboratories, and show data suggesting a pattern of inadequate pain management in laboratories coupled with incomplete pain management reporting in scientific journals. We will discuss the ethical and scientific implications of these findings and make recommendations to address the problem of pain in animal experimentation.

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## Development of an optimized culture medium to differentiate MUTZ-3 cells into MUTZ-Langerhans cells for *in vitro* skin sensitization assays

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Langerhans cells (LCs), the dendritic cells of the skin, play an important role in the immune response. Thus, dendritic cells can be considered important components in the development of *in vitro* tests intended to assess the skin-sensitizing potential/potency of chemicals. However, due to limited supply and donor variability of native LCs isolated from human skin, surrogate cell lines are commonly used. One known surrogate is the human acute myeloid leukemia cell line MUTZ-3, which resembles the phenotype and morphology of human CD34-positive dendritic cell precursors. Upon exposure to a cytokine cocktail the MUTZ-3 cells differentiate into Langerhans-like cells (MUTZ-LCs), a prerequisite to elicit LC-typical reactions upon exposure to potential sensitizers. For that, two aspects must be considered: the composition of the differentiation medium and the differentiation time.

Besides defined cytokines like GM-CSF, TGF- $\beta$  and TNF- $\alpha$ , the majority of published differentiation media are still supplemented with undefined cytokine cocktails like the 5637 bladder carcinoma-conditioned medium (5637CM) and fetal calf serum (FCS). Both supplements are also prone to lot-to-lot variabilities. Hence, our goal was to improve the standardization of the MUTZ-3 cell differentiation protocol, while retaining all LC-typical expression markers, which were analyzed by flow cytometry.

Our results demonstrate that 5637CM can be completely excluded, as the expression of pivotal proteins like CD1a or CD207 remains unchanged. Moreover, when reducing FCS percentage from originally 20% to less than 10%, the desired differentiation status was already achieved after 7 days instead of 14 days of culture in the majority of cells. Hence, the differentiation time could be markedly reduced.

In conclusion, our optimized differentiation protocol requires less time and materials and has a higher degree of standardization, as the medium is free of undefined 5637-conditioned medium and is only supplemented with a reduced FCS amount.

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## The GARDpotency assay for potency-associated subclassification of chemical skin sensitizers – Rationale, method development and ring trial results of predictive performance and reproducibility

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The advancement of non-animal approaches for hazard assessment of skin sensitizers have generated a variety of alternative assays with discriminatory properties comparable with those of accepted *in vivo* methods. However, hazard identification is rarely sufficient and information permitting the relative ranking of chemicals' skin sensitization potency is desired. For example, the globally harmonized system of classification and labelling of chemicals (GHS/CLP) extends the binary hazard assessment with a qualitative sub-categorization to distinguish between weak and strong skin sensitizers.

Though substantial efforts have been made towards developing alternative methods for potency assessment, none have gained regulatory acceptance, emphasizing that continued development of improved alternative assays remains a high priority.

The genomic allergen rapid detection (GARD) is an *in vitro* testing platform for assessment and characterization of chemical sensitizers, based on evaluation of transcriptional patterns of endpoint-specific genomic biomarker signatures in a human dendritic-like cell line following chemical exposure, in order to provide machine learning-assisted classifications of tested substances. The GARDskin assay was recently subjected to a formal validation procedure (OECD TGP 4.106) and reported a reproducibility between laboratories of 92%, as well as a predictive accuracy of 94%, for sensitization hazard assessment.

Here, we present the implementation of the related GARD application GARDpotency, for potency-associated subcategorization of chemical sensitizers. Following prediction model establishment, the functionality of the assay was validated in a blinded ring-trial, in accordance with OECD-guidance documents, by assessing predictive performance and reproducibility. It was found that the assay is functional and predictive, with an estimated cumulative accuracy of 88% across three laboratories and nine independent experiments. The within-laboratory reproducibility measures ranged between 63-89%, and the between-laboratory reproducibility was estimated to 61%. In conclusion, the *in vitro* GARDpotency assay constitute a standardized, functional assay, which could be a valuable tool for hazard characterization of skin sensitizer potency.

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## OncoChip: Development of a microfluidic human test platform for immune-oncology therapy testing

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Modern immune-oncology therapies aim at treatments that take advantage of the body's immune system to fight cancer. The efficacy of these therapies combines orally administered chemical oncology drugs together with systemically injected monoclonal antibodies. Antibody-dependent cell-mediated cytotoxicity (ADCC) is an immune mechanism enabling Fc receptor-bearing effector cells, mainly natural killer cells (Nk cells), to recognize and kill tumor cells, which carry antibody-coated tumor antigens on their surface. No laboratory animals nor current *in vitro* testing tools represent the human immune-oncological background of patients.

The objective of this project is to establish a human test system for pre-clinical immune-oncology therapy testing. Therefore, the HUMIMIC Chip2, a commercially available microfluidic multi-organ chip platform, is used. The establishment of a long-term co-culture of a human intestinal equivalent and a tumor-infiltrated liver model interconnected through a microfluidic vasculature on this chip is in progress. To guarantee an autologous system induced pluripotent stem cells of one donor are used. Furthermore, primary NK cells are added to the circulation to mimic effector cell distribution. The intestinal equivalent and the vascularized circuit support "oral" and "intravenous" administration of immune-oncology drugs, respectively. The tumor is modelled by H292 cell line spheroids overexpressing EGFR. This receptor is recognized by the monoclonal antibody Cetuximab which in turn attracts primary natural killer cells via its Fc-part to lyse the tumor via the perforin/granzyme cell death pathway. NK cell specific tumor cell killing on the background of a functional intestine-liver-vasculature co-culture is envisaged to prove the assay concept.

We summarize the results of the experiments with the readouts being flow cytometry analysis of NK cells, metabolic activity assays including LDH release, glucose consumption and lactate production, immunohistochemical analysis and specialized ADCC-apoptosis assays. Finally, we propose a qualification program for the OncoChip assay for industrial adoption to internal preclinical oncology portfolio decision-making.

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## Toxicity testing in an *in vitro* model for anti-carcinogenesis demonstrates the improved preventive effects of combinations of bioactive compounds as compared to single compounds

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The goal of toxicity testing is to ensure appropriate protection of public health from the adverse effects of exposures to environmental agents. However, these toxicity tests can also be used to evaluate the possible protective effects of different types of exposures on human health. In the present study, we demonstrate the value of toxicity testing in a colonic *in-vitro* model for anti-carcinogenesis, which was validated against a previously conducted human dietary intervention study on blueberry-apple juice. Blueberries contain a mix of different phytochemicals which might be responsible for their disease preventive properties. In the human study, we showed that a 4-week intervention with blueberry-apple juice protected the participants against *ex-vivo* induced oxidative stress and modulated expression of genes involved in different genetic pathways contributing to the antioxidant response. The *in-vitro* study investigated the effect of different blueberry varieties and the blueberry-apple juice from our previous human dietary intervention study, and four different single compounds on antioxidant capacity and gene expression changes in colonic cells *in-vitro* and compares the outcome with the earlier *in-vivo* findings. Protection against oxidative stress was measured via the Comet assay, which detects DNA strand breaks. A reduction in the level of induced DNA strand breaks is regarded as a preventive effect. The results demonstrate that all blueberry varieties as well as the blueberry-apple juice were more effective in reducing oxidative stress induced DNA strand breaks as compared to the single compounds. In addition, the gene expression profiles induced by the blueberry varieties were more similar to the profile of the human intervention study than the single compounds. These results indicate the added value of toxicity testing for identifying combinations of phytochemicals which show optimal preventive capacities. Applying this approach in innovative organoid-based *in-vitro* models may further advance this field of research.

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## The rodent in the room: Considering sentience in research programs using mice and rats

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Animal sentience – the ability to feel emotions such as joy, pain, fear, suffering, and happiness – is an inherent reason for ethical discussion surrounding the use of animals in research, however, the topic itself is often overlooked. This occurs despite a growing body of scientific evidence attesting to the rich inner lives of animals commonly used in research. Rats and mice are the most frequent animal species used in experiments, with an estimated 100 million killed annually in the United States alone. There are numerous published studies demonstrating that mice and rats experience emotions. Moreover, many biomedical, behavioral, and psychological experiments on these rodents are predicated on their sentience. Indeed, the ability of animals to experience complex emotions is a necessary basis for any attempts at translating data from these studies on animals to humans. Rats have been documented displaying empathy for other rats, committing acts of altruism, engaging in play and other forms of joy, regretting poor choices, and working to free other rats who are trapped. Mice have also demonstrated empathy and will work to free trapped conspecifics. Nevertheless, this knowledge, though widespread and readily available, seldom translates into considerations of sentience when experiments are being designed and approved. I suggest that the knowledge gap between the current science on sentience, the researchers who work with mice and rats, and the oversight committees that approve experiments has resulted in an overly permissive attitude toward the use of mice and rats in experiments. Through a literature review, my research describes the current state of science on the emotional experiences of mice and rats, opens a vital discussion about the unexamined role of animal sentience in welfare considerations and experimental design, and provides strategies to incorporate considerations of animal sentience into research programs to promote better science and improve animal welfare.

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## Long term culture for repeated dose assay: Are 3D models relevant?

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Shelf life for 3D models has always been an open question. To be more accurate what users have been asking is the possibility to keep them longer than the original shelf life. The interest of keeping the tissues several weeks could be the evaluation of the effect of multiple and long-term application for specific products and initiate a chronic like assay. But it is necessary to be confident on tissues viability and scientific relevancy all along the assay.

In this study we were able to keep the SkinEthic RHE model in test condition for 4 weeks (D17 to D45). Each week, we assessed different endpoints to evaluate the quality of the epidermis:

- ET50 to evaluate the barrier function,
- Histology to observe the structure of the epidermis
- Negative and Positive control of OECD TG439 test to evaluate the potency of the epidermis to respond to an irritant chemical.

This assay is really encouraging and showed that RHE model can be cultivate longer than the original shelf-life.

The structure of the epidermis was quite satisfactory, the barrier function was still effective, and the model was able to discriminate negative and positive controls.

Taken into account these promising results, further investigations are needed such as test of the chemicals of the proficiency list of the OECD TG439, analysis of different markers of proliferation, differentiation and inflammatory, etc. (KI67, Filaggrin, Involucrin, Cytokeratin 10, Transglutaminase 1).

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## Reliable and truly animal-free skin sensitization testing – Adaption of the *in vitro* GARDskin assay to animal-free conditions

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A plethora of *in vitro* approaches for hazard assessment of skin sensitizers have recently been described and demonstrated to exhibit discriminatory properties competitive with those of accepted *in vivo* methods. However, the majority of these *in vitro* meth-

ods still use animal-derived components such as fetal calf serum (FCS) within their standard protocols, imposing the question whether these methods should truly be considered as animal-free replacements.

Genomic Allergen Rapid Detection™ – GARD™ – is a genomics-based *in vitro* testing platform for assessment of numerous immunotoxicity endpoints. The endpoint-specific classification of skin sensitizers is a well-established application of the platform, referred to as GARD™skin (OECD TGP 4.106). The assay is based on a human DC-like cell line (SenzaCells™) and utilizes machine learning to classify chemicals by monitoring the expression of 200 genes involved in cellular pathways associated with skin sensitization. GARD™skin is progressing towards regulatory acceptance, and consistently reports accuracies > 90%.

Here, we present an adaption of GARDskin standard protocol to enable for testing under animal-product-free conditions by replacing animal-based FCS with human derived serum. SenzaCells adapted well to routine culture in the human serum, showing comparable cell viability and growth rates to the animal-based FCS. A phenotypic analysis of common DC maturity markers showed minor changes in cell surface expression of the markers CD14 and CD1a, indicating that serum replacement did not significantly alter the phenotypic characteristics of the cells. Finally, a proficiency set of nine chemicals covering the full range from extreme sensitizers to non-sensitizers were evaluated. The protocol adapted to animal-free conditions showed full concordance to the conventional protocol, correctly classifying all chemicals.

In conclusion, this demonstrates the potential to perform GARDskin without the use of animal-derived components associated with animal welfare concerns, thus paving the way for truly animal-free and highly accurate hazard testing of skin sensitizers.

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## Hazard assessment of photoallergens using GARD™skin

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Chemicals of different categories, such as cosmetics and drugs, have the potential to become photoactivated when exposed to UV light, giving rise to otherwise dormant adverse effects such as sensitization. Such chemicals, referred to as photoallergens, causes a Type IV delayed hypersensitivity, typically manifested as allergic contact dermatitis. While *in vitro* assays for prediction of a chemical's potential to provoke phototoxicity (photoirritation) have been proposed, there is no recognized assay that specifically predicts photoallergens. Therefore, development of



accurate *in vitro* assays that can detect photoallergens remains a high priority.

The Genomic Allergen Rapid Detection™ – GARD™ – platform constitutes a unique framework for classification of numerous immunotoxicity endpoints. The endpoint-specific classification of skin sensitizing chemicals is a well-established application of the platform, referred to as the GARDskin assay. GARDskin utilizes the readout of a genomic biomarker signature of 200 genes, which allows for machine learning-assisted classification of skin sensitizers. The assay is progressing towards regulatory acceptance and demonstrates high predictive performance.

Here, we present an adaptation of GARD protocols, allowing for assessment of chemical photoallergens. By incorporating UVA exposure during sample preparation, photoactivation of latent photoallergens has been demonstrated. In a first step, protocols were optimized using the photoallergens 6-methylcoumarin and Ketoprofen, exposed to UVA light, both prior to and in association with cellular exposure, along with appropriate radiated/non-radiated controls. Photoallergenicity was accurately predicted in both test chemicals exposed to UVA light, while non-radiated counterparts were accurately classified as non-sensitizers.

In summary, our initial data demonstrates a potential of GARDskin to assess photoallergenicity of chemicals. Further evaluation and optimization of the method are currently in progress, in which an extended panel of fragrances are being studied in a collaboration with the Research Institute for Fragrance Materials (RIFM).

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## Practical application of *in silico* approaches in next generation risk assessment for consumer products

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Use of computational models is commonplace across the consumer product development pipeline. Approaches include Structure-Activity Relationships, Quantitative Structure-Activity Relationships, read-across, as well as mechanistic and pharmacokinetic models. Next Generation Risk Assessments should be exposure-led and quantitative, so that predictions relating to dose may be interpretable in the context of exposure. This allows methods to identify and characterize hazard and risk to be applied in a tiered manner starting with exposure-based waiving and building through *in silico*, *in chemico* and *in vitro* assays combined in weight of evidence assessments (Dent et al., 2018) The need for robustness, reliability and traceability of decisions made using computational approaches is paramount to their acceptance and success.

When attempting to complete a risk assessment including computational approaches it is often necessary to generate additional data using so-called New Approach Methodologies to build confidence in the underlying hypothesis. Indeed, the use of read-across can often help to define a hypothesis that leads to further testing, e.g., *in vitro* point of departure characterization, or metabolism investigation. In this way computational approaches help to structure an assessment.

*In silico* approaches are used by many regulators for prioritization and screening of chemical inventories and there are some limited examples of use in final safety decisions. Regulatory uptake of *in silico* approaches remains a challenge in some areas (e.g., chemical registration), though they are often listed as potential alternative approaches. Some chemical regulations are hazard-based and do not include considerations of exposure and risk. More flexibility regarding the inclusion of non-traditional data and alternative means of demonstrating safety for chemicals registrations together with sharing examples of scientifically robust assessments will help to increase the acceptance of read-across and *in silico* approaches to allow them to fulfil their potential to drastically reduce the reliance on animal testing.

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## Framework for physiologically based kinetics (PBK) modelling in the next generation risk assessment (NGRA) of consumer goods

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Physiologically based kinetics (PBK) modelling is an integral part of the tool set used in Next Generation Risk Assessment of ingredients in consumer products (Moxon et al., 2020). Accurate predictions of the exposure allow for comparison to biological effects, and an understanding of the risk to consumers. But providing confidence in these exposure predictions without the use of *in vivo* data for validation can prove difficult. This work proposes and outlines the use of a PBK framework, with application to a number of case study chemicals (caffeine, coumarin and sulforaphane) in hypothetical products.



The framework highlights how confidence in PBK model can be increased through an iterative process, and by using sensitivity analysis to identify parameters which will have a large influence on the model output. The use of Monte Carlo analysis allows for population variability and parameter uncertainty to be integrated into the study, and a distribution of C<sub>max</sub> values calculated, instead of a point estimate.

The case studies show that the framework can be applied to a variety of products whose main route of exposure is transdermal, and in cases where the product is left on the skin (e.g., face cream and body lotion) or rinsed-off (e.g., shampoo and kitchen cleaner). The work highlights the need for running the simulation for sufficient amounts of time during repeat exposure to ensure that steady-state has been reached (up to 20 days of repeat exposure in some cases). Validation of the models against published clinical trial information shows that they predict within 3-fold of the observed C<sub>max</sub> results, without the use of animal/human data.

#### Reference

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### **An *in vitro* microfluidic model of the human cardiovascular system for use in screening applications – Assessment of monocyte-to-endothelial cell adhesion and cytokine analysis**

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Atherosclerosis (gradual narrowing of the arteries) is an inflammatory process involving a series of progressive cellular events, including monocyte adhesion to the luminal endothelial surface. We have developed an *in vitro* cardiovascular adhesion assay using BioFlux™ microfluidic technology to model monocyte adhesion to primary human aortic endothelial cells (HAECs), an important early stage of atherosclerosis and predictive of *in vivo* inflammatory outcomes. HAECs lining microfluidic channels are exposed to shear flow, mimicking blood vessel physiology and blood flow through the vasculature. Our work provides proof of concept data to establish assay sensitivity for detection of pro- or anti-inflammatory effects of chemicals, pharmaceuticals or food compounds on the endothelium. This model can detect changes in cytokine secretion in response to known inflammatory media-

tors and provides an *in vitro* endpoint for screening and pre-clinical assessment as a predictor of atherosclerotic risk. The effect of aspirin, lipopolysaccharides (LPS), clopidogrel, doxorubicin, methotrexate, 1-chloro-2,4-dinitrobenzene (DNCB), cobalt chloride and 5-hydroxymethylfurfural (5-HMF) on monocyte adhesion to HAECs was evaluated for pro- or anti-inflammatory outcomes which can be mechanistically linked to modulation of cytokine release. Endothelial monolayers were grown in microfluidic channels and exposed to test compound. HAEC treatment with TNF $\alpha$  was used to induce a pro-inflammatory phenotype. Activated human monocytic leukaemia (THP-1) cells were perfused through the microfluidic channels and a perfusion, adhesion and wash cycle performed with increasing adhesion period length. Monocyte adhesion was quantified and after the final adhesion and wash steps cytokine (IL-6, IL-10, IL-1 $\beta$ , TNF $\alpha$ , IL-8, IL-12p70) secretion assessed by bead array flow cytometry. Using this technology, we have developed a physiologically relevant *in vitro* assay to assess atherosclerotic/immunological risk that provides a bridge between *in vitro* and *in vivo* experiments.

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### **Next generation risk assessment for skin allergy: 0.1% coumarin in face cream *ab initio* case study**

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Next Generation Risk Assessment (NGRA) is an exposure-led, hypothesis-driven approach integrating new approach methodologies (NAMs) to ensure the safety of consumer products without animal data. We have applied an *ab initio* tiered framework based upon ICCR principles (Berggren et al., 2017; Dent et al., 2018) to a hypothetical skin allergy risk assessment, where 0.1% coumarin is used in a face cream. For the purpose of evaluating the use of NAMs, existing animal and human data for coumarin were excluded.

Initially, applied dose exposure estimates for coumarin were used to determine the extent of skin exposure and *in silico* chemistry predictions (ToxTree, OECD Toolbox) were used in conjunction with expert opinion to establish skin allergy potential. Then the skin allergy risk assessment was conducted using DPRA (OECD TG 442C), KeratinoSens™ (OECD TG



442D), h-CLAT (OECD TG 442E) and U-Sens™ (OECD TG 442E) data were generated and a point of departure (PoD) derived from our Skin Allergy Risk Assessment (SARA) model / defined approach (DA; Reynolds et al., 2019). By evaluating the SARA DA prediction for 0.1% coumarin in a face cream against other skin allergy risk benchmarks we can conclude that there is a low risk of inducing skin allergy under this exposure scenario.

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## A microfluidic thyroid-liver platform to investigate mechanisms of thyroid toxicity in humans and rats

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The assessment of the human relevance of toxicities observed in animal assays still represents a major challenge for various areas of toxicology. Rodents, which are widely used for regulatory toxicity testing, are particularly sensitive to perturbations of the thyroid homeostasis. Evaluation of the species-specificity of adverse effects on the thyroid gland is key for the regulatory acceptance of a risk assessment. Thyroid toxicity may result from direct effects on the thyroid gland or be mediated, e.g., by inducing liver biotransformation. Microfluidic microphysiological systems (MPS) represent a promising approach to reproduce physiological and toxicological interactions of target organs which previously required animal studies or clinical trials. However, adoption of MPS by the pharmaceutical or agrochemical industry is still slow, mainly driven by a lack of qualified assays to predict the safety of drugs and chemicals. Here, we present an interconnection of three-dimensional organ models representing two important target organs, liver and thyroid, of both human and rat origin in a commercially available multi-

organ-chip platform. The structural and functional integrity of both organoids could reproducibly be demonstrated for at least 14 days, i.e., by steady glucose consumption, low LDH release, immunohistochemistry of thyroid follicles and liver spheroids, and organ-specific functional readouts like albumin secretion, urea production and thyroid hormone release by liver spheroids and thyroid follicles, respectively. Finally, perturbation of the hepatic thyroid hormone catabolism was demonstrated by acceleration of thyroid hormone glucuronidation following treatment with reference inducers like beta-naphthoflavone. Thus, we show for the first time a functional model of the hepatic-thyroid axis as single *in vitro* assay for two different species. These two chip-based models represent a major step towards an improved assessment of potential species similarities/differences of certain toxicity findings observed in rodents with significant contributions to the 3R principles.

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## A perfect match reduces animal use

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Zoology is one of the specialization courses of Life Sciences program at the University of Applied Sciences in Utrecht. This specialization trains to become an HBO-animal biotechnician. The program had a huge influx of students in recent years (± 300 students). On average, 25 students start each year in the Zoology specialization. In this phase, students learn to approach tissues in a correct way on dead rats. Thereafter, students learn to handle, restrain and inject living rats and mice. They also perform surgical procedures including suturing on sedated animals. Subsequently, these techniques are applied in several experiments. Here, implementation of skills, a good pace of work and care of animal welfare are important learning objectives. Twelve animals are used per student to demonstrate their prerequisite competences to become a high-quality future HBO-animal biotechnician.

To handle the large influx of students, we have been applying a stricter matching process for the past two years. We aim to prevent early drop-out due to emotional/ethical reasons. Next to an introductory zoology course in the general phase of the educational program, this matching consists of 1) writing a motivational letter in which the students substantiate their specialization choice, 2) having a motivational interview with the lecturers involved, 3) attending a practical experiment of the Zoology specialization phase and 4) visiting an animal experimental labora-



tory. The aim of this approach is to achieve that students make a more considered choice of specialization.

As a result of this matching process, eight students ultimately chose a different specialization. Thanks to this intensive process, we were not only able to give students a better picture of the zoology educational program and their future line of work as an HBO animal biotechnician, but we were also able to reduce animal use by nearly 100 animals in two years.

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## Development of mechanism-based hematotoxicity categories for read-across assessment using an integrated toxicity database of chemical substances

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Read-across is one of the techniques for filling data gaps by grouping a target substance and the tested analog(s) for chemical safety assessment and regulatory decisions. To perform read-across assessment for chemicals with the potential of inducing hemolytic anemia, a category approach was explored using test data from major publicly available toxicity databases and mode of action information. Firstly, an integrated database was constructed by collecting repeated dose toxicity datasets from HESS, ToxRef, COSMOS, and RepDose. In the database, 447 of 1550 substances were identified as those of hematotoxicity that induced hemolytic anemia. Secondly, grouping based on chemical structures and toxicity mechanisms of the hemolytic substances led to the following categories: anilines (Category 1), nitrobenzenes (Category 2), nitroanilines (Category 3), hydrazines (Category 4), oximes (Category 5), and ethylene glycol alkyl ethers (Category 6). Using the integrated toxicity database, a more extensive range of category members has been obtained and the structural boundaries of some categories have become clearer. Moreover, most of these category substances produced hemolytic effects as a critical one which is likely to determine the point of departure. Finally, the mechanisms of hematotoxicity were postulated that: reactive metabolites of substances in Categories 1 to 3 oxidize hemoglobin; substances in Categories 4 and 5 directly oxidize hemoglobin; and reactive metabolites of Category 6 substances modify erythrocyte membrane. Such metabolic and biological information is measurable or predictable, and critical to justify the categories. Given the larger toxicity dataset, and information on the mechanistic key event linking to the toxicity end-

point, these categories could support read-across that has the improved confidence for the regulatory safety assessment.

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## Successful implementation of U-SENS™ assay for skin sensitization testing (OECD TG442E) in China

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The U-SENS™ assay is an *in vitro* method to assess skin sensitization, addressing the third key event (KE3) of the skin sensitization Adverse Outcome Pathway, the activation of dendritic cell following exposure to sensitizers was modelled in the U937 cell line by measuring the expression of CD86 by flow cytometry. This assay, recommended to be used as part of an integrated approach for risk assessment, has been adopted by OECD Test Guideline 442E as part of the Mutual Acceptance of Data (MAD), a multilateral agreement which allows participating countries (including non-members) to share the results done on chemicals using OECD methods and principles. In China, considering the high interest of development and implementation of non-animal alternative methods for sensitization assessment, several authority laboratories and testing service entities expressed their interests in developing *in vitro* testing strategies integrating U-SENS™ as KE3 method.

This study aims to implement the U-SENS™ test method to build up the qualified scientific capacity and expertise in China, for further method appropriation to Chinese laboratories and the development IATA strategy for skin sensitization.

Through a formal training program consisting of the demonstration of reproducible positive results for abietic acid, and consistent negative results for lactic acid, Chinese scientists were qualified. During the internal validation, the scientists independently performed the U-SENS™ assay for 6 recommended OECD TG 442E reference chemicals. All chemicals were correctly classified, demonstrating the expertise and knowledge appropriation of the method by the scientists that are therefore considered as a successful transfer.

In conclusion, the U-SENS™ test method was successfully implemented in China and considered in Chinese authority laboratories and testing services organizations for implementation. Appropriation of the U-SENS™ test method to China definitely promotes the non-animal testing methods for skin sensitization assessment, significantly contributes to the development of integrated testing strategies for testing needs.

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## A ready-to-use integrated *in vitro* skin corrosion and irritation testing strategy using EpiSkin™ model in China

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The need of *in vitro* alternative methods has been increasing in toxicology research as well as in cosmetic industry in China recently. Currently, regulatory agencies require acute cutaneous hazard assessment using the Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS). Following the establishment of EpiSkin™ skin corrosion and irritation *in vitro* tests in China, both as stand-alone methods according to Organization for Economic Co-operation and Development (OECD) TG 431 and TG 439, their combination within the Integrated Approach on Testing and Assessment (IATA adopted as OECD Guidance Document 203) could be considered in a modular approach.

The present study exemplifies the application of both methods in a bottom-up integrated testing strategy with specific case studies on three chemicals. A chemical (Isopropanol, CASRN 67-63-0) known to be No Cat. *in vivo*, was predicted as No Cat. in the first tier strategy using EpiSkin™ skin irritation test method. Being identified as classified (not No Cat.) in the first step (TG 439), both 1-Bromohexane (*in vivo* Cat. 2; CASRN 111-25-1) and lactic acid (*in vivo* Cat. 1B/1C; CASRN 598-82-3) were further assessed in the second-tier step which consists of their evaluation using EpiSkin™ skin corrosion test method. 1-Bromohexane identified as a Not Cat.1 was therefore classified as Cat.2, whereas lactic acid identified as a Cat.1 was even subcategorized as a Cat. 1B/1C. These case studies reflect an approach on how to move from animal testing into an evaluation of new ingredients.

In conclusion, the integration of China EpiSkin™ skin corrosion and irritation testing data into either bottom-up or top-down strategy allows accurate assessment of potential skin hazard of chemicals. It brings a future extension of application of alternative methods and implementation of alternative testing strategies in China.

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## Multi-center validation of EpiSkin™ micronucleus assay: A 3D approach for the assessment of genotoxicity potential

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The establishment of *in vitro* genotoxicity assessment using 3D skin models becomes essential for the safety evaluation of new chemical with topical exposure to overcome the limitations of 2D assays. Cosmetics Europe established a 3D micronucleus assay using EpiDerm™ model. To meet the increasing testing requests especially for Asian needs, in China, a novel *in vitro* human reconstructed skin micronucleus assay has been developed using locally produced Episkin™ model (Chen et al., 2020). The Episkin™ Micronucleus Assay showed good predictivity and reproducibility during the internal validation. In this study, a formal multi-center validation was conducted in China with 3 laboratories. 28 reference chemicals were selected by Cosmetic Europe Genotoxicity Taskforce with different physicochemical properties and coded by the third-party organization. 14 of them were blind tested in all three laboratories, then the other 14 were randomly assigned to the labs. Independent statistical expert performed the decoding, data analysis and released the report. Auditor with GLP credential checked the document managements and quality management systems of each participating organization.

The report of the first 14 chemicals has been released. Data of all batches met quality criterion described in SOP, which indicated the stability of model production and scientists' operation. Classification results showed good sensitivity (85.7%) and specificity (85.7%) for its predictive capability of genotoxicity potential.

Altogether, these results showed that micronucleus assay using reconstructed skin model offered promising tool for the genotoxicity assessment of chemicals applied on skin. The availability and validity of the Episkin™ Micronucleus Assay are expected to contribute to the *in vitro* genotoxicity testing strategy to follow up on positive results from standard 2D *in vitro* assays. This validation study will strongly support the com-



mitment to develop alternative methods to animal testing and the safety assessment needs for industry and regulatory purpose.

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### Implementation of alternative methods in China according to OECD Guidance Document on Good In Vitro Method Practices: An example

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An OECD Guidance Document No. 286 on Good In Vitro Method Practices (GIVIMP) for the development and implementation of *in-vitro* methods for regulatory use in human safety assessment was recently endorsed. In China, this is therefore primordial for the implementation and acceptance of *in-vitro* alternative methods.

Since 2011, L'Oréal initiated the EpiSkin™ Skin Irritation Test (SIT) implementation program in China. 69 scientists received formal trainings and the method has been established in 39 laboratories including industrial, authoritative and research organizations. Taking one of them, the multi-center study of SIT, as example, we demonstrated a standard method establishment process in good alignment with GIVIMP.

The quality of EpiSkin™ was assured by Shanghai EPISKIN Biotech providing relevant safety information for transport, use and disposal (GIVIMP 1.2, 5.3). All tested chemicals, commercially available from certified suppliers, were selected from publications that supported the adoption of the method into TG 439 (GIVIMP 4.2, 6.1, 8.4). The formal step-by-step training according to SOP was provided to 4 labs (GIVIMP 1.1, 2.6, 7.1, 7.2, 8.2) which conducted the MCS together with L'Oréal. By showing qualified results of training set chemicals, scientists were allowed to conduct the MCS of 20 reference chemicals described in TG 439. A powerful and simple statistic tool, and control trend charts were used to monitor reproducibility (GIVIMP 2.3, 2.4), showing >90% within-lab reproducibility in all labs and 95% inter-lab reproducibility (GIVIMP 8.3). The overall predictive capacity including 70% specificity, 94% sensitivity and 82% accuracy met the criteria defined in TG 439 (GIVIMP 8.3, 9.5).

In conclusion, the EpiSkin™ SIT implementation program in China, exemplifies the practical way in which the GIVIMP can assist interested parties in the transfer and establishment of *in-vitro* approaches, and also pave the way towards future scientific recognition and acceptance of *in-vitro* alternative methods in this fast-developing country.

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### Development of a 3D skin Comet assay: Building the foundation for higher tier *in vitro* testing battery of genotoxicity

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Genotoxicity evaluation is mandatory for safety assessment of cosmetic ingredients. It is generally addressed by using a battery of standard *in vitro* assays covering gene mutations, structural and numerical chromosome damage and of *in vivo* assays as higher tier to follow up on positive outcomes for regulatory purposes. In the frame of our commitment for animal testing ban, we targeted our research for the development of a high-tier *in vitro* genotoxicity assays. Given a 3D micronucleus assay previously developed in the follow-up strategy (Chen et al., 2020), we propose another test based on reconstructed skin models (3D skin), the skin comet assay to construct the higher tier *in vitro* testing battery.

Our strategy is to propose and validate the 3D skin comet assay as one of the “2<sup>nd</sup> tier” to address the mutagenic endpoint of chemicals. The protocol was firstly set up with two model genotoxins (4NQO and MMS). A stable baseline level, less than 20% tail intensity, was established to extrapolate positive responses. The intra-lab reproducibility was further demonstrated with additional 4 genotoxic carcinogens at non-cytotoxic range of concentrations and the results showed good concordance with *in vivo* data. Our data show that comet assays using reconstructed skin models offer promising results for the genotoxicity assessment of chemicals with a dermal route of exposure. The transferability and validity of 3D skin comet assay will be further evaluated in the near future. Overall, the 3D skin comet assay presents a strong candidate as “2<sup>nd</sup> tier” assays in the follow-up strategy. Meanwhile this study is the first in China to establish higher tier *in vitro* assays using locally produced full thickness skin model. It is an important stage in our commitment for the development of alternative methods for the safety evaluation of our products.



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Chen, L., Li, N., Liu, Y. et al. (2020). A new 3D model for genotoxicity assessment: EpiSkin™ micronucleus assay. *Mutagenesis* 36, 51-61. doi:10.1093/mutage/geaa003

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## Full replacement of regulatory skin sensitization testing with validated *in vitro* tests: Integrating the kinetic peptide reactivity assay into the test battery

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Several *in vitro* tests addressing key events 1-3 of the adverse outcome pathway for skin sensitization have been adopted as OECD guidelines, but no test had been formally validated for sub-classification into the potency classes GHS 1A and GHS1B, a regulatory need today still met by animal testing. The reaction of sensitizing molecules with skin proteins is the molecular initiating event. It appears to be a rate limiting step and thus an important determining factor for sensitizing potency. The kinetic direct peptide reactivity assay (kDPRA) (Roberts and Natsch, 2009; Wareing et al., 2017) allows deriving rate constants of the reactivity of test chemicals with a cysteine-containing model peptide. Reproducibility was recently proven in an inter-laboratory study in seven laboratories. Based on a database of rate constants for 180 chemicals, an optimal prediction cut-off to identify strong sensitizers (GHS 1A) was derived, which has a balanced accuracy of 85% vs. the local lymph node assay. The kDPRA is thus proposed as an assay for GHS sub-classification. It can be combined with a scheme for hazard identification such as the 2 out of 3 approach (Bauch et al., 2012): With two key event tests positive, a chemical is rated as sensitizer. A subsequent kDPRA then attributes the chemical to a GHS potency class. This is the first testing strategy only based on formally validated *in vitro* endpoints providing a full replacement of regulatory skin sensitization testing. Here we discuss the details of this strategy, validation status, applicability domain and predictivity.

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## Defining physicochemical exclusion rules to identify chemicals that do not require classification of serious eye damage/eye irritation: A Cosmetics Europe analysis

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Cosmetics Europe's (CE) ocular toxicity program focuses on the development of defined approaches (DA) to identify ocular effects of chemicals (in the context of OECD's Guidance Document on an Integrated Approach on Testing and Assessment for Serious Eye Damage/Eye Irritation). CE created a comprehensive database of chemicals for which *in vitro* data are available with corresponding historical *in vivo* Draize eye data and physicochemical properties (PCP). One key outcome of this project is that incorporating specific PCP exclusion rules (based on water solubility, LogP, vapor pressure, surface tension) in a DA resulted in an increase of the specificity without affecting the sensitivity of the stepwise approach.

PCP's of 160 liquids were retrieved from the following sources: the European Chemicals Agency (ECHA) website, the Environmental Protection Agency (EPA) Chemistry Dashboard website, the PubChem website, and the ChemSpider website. Highest priority was given to experimentally derived measurements followed by computational methods (e.g., Quantitative Structure-Activity Relationships) used to determine PCP's. For each PCP a comparison was performed between different data sources.

None of the GHS Cat. 1 liquids (n = 21) and none of the GHS Cat. 2 liquids (n = 26) did meet the PCP exclusion rules (0 false negatives). On the other hand, the PCP exclusion rules were met for 51/113 GHS No Cat. liquids resulting in a true negative pre-



diction. To identify GHS No Cat., the combination of PCP with RhCE resulted in an increase of the specificity of on average 15% (from 61% by RhCE only to 76% by combination of RhCE with PCP exclusion rules). The impact of PCP exclusion rules on the performance of the DA is illustrated in violin plots with indication of the corresponding predictions. In conclusion, PCP exclusion rules are valuable tools to support the identification of GHS No Cat. without decreasing the sensitivity of the DA.

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## Zebrafish embryo model for chemical-induced craniofacial anomalies

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Various alternative methods for testing the developmental toxicity of chemicals have been developed worldwide. Zebrafish embryos are non-protected animals and represent a promising model for assessing teratogenicity owing to their rapid development and transparency. Craniofacial anomalies are among the most frequent birth defects and considered to be a critical endpoint for evaluating chemical-induced teratogenicity. However, the conserved developmental process(es) disturbed by teratogens in zebrafish and mammals has not been well studied.

In the present study, zebrafish embryos were treated with 12 chemicals known to be teratogens in mammals. Cartilage staining with Alcian blue was performed to examine detailed craniofacial anomalies and for morphological quantification of chondrocyte defects. Also, comprehensive and semi-quantitative gene expression analyses were performed to examine the conserved biological pathways between zebrafish and mammals.

Detailed observations showed that all 12 teratogens induced craniofacial malformations in the zebrafish neurocranium and viscerocranium. Further observations revealed characteristic disruption of chondrocyte proliferation, differentiation, and maturation. These findings suggested a failure of cranial neural crest (CNC) cell development, which was confirmed by gene expression analysis of the CNC. Furthermore, several of these chemicals caused malformations of the eyes, otic vesicle, and/or heart, representing a phenocopy of neurocristopathy, i.e., human diseases associated with abnormal neural crest development. These chemicals altered the expression levels of the responsible genes.

These observations paralleled the abnormalities reported to be caused by these chemicals in mammals, suggesting conserved etiological pathways between zebrafish and mammals. We showed that disruption of CNC development is the target biolog-

ical pathway underlying the chemical-induced craniofacial malformations, a finding that represents a promising molecular endpoint for mechanism-based teratogenicity evaluation. This study established that zebrafish represent a suitable *in vitro* model for teratogenicity assessment as well as screening tool for prediction of craniofacial anomalies in mammals (Liu et al., 2020).

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**Presentation:** Poster

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## *In vitro* evaluation of genotoxicity and irritation potential of eye drops containing aqueous plant extracts

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According to Committee on Herbal Medicinal Products (HMPC) Guidelines, the genotoxic potential of herbal preparations shall be assessed since genotoxic effects cannot be completely ruled out based on pharmacovigilance and long-standing use only. Also, in a product specific risk assessment further gaps in non-clinical data, e.g., local tolerance testing for the final pharmaceutical product should be taken into account. Following the R3 principles, there is a drive to omit *in vivo* testing whilst meeting the requirements of safety evaluation. Although *in vitro* methods accepted by OECD are available, these are mainly applied to single chemical substances. Yet herbal preparations are multicomponent mixtures, reaching an even higher level of complexity when used in combinations. Therefore, the study aimed to assess the applicability of *in vitro* tests to single plant extracts as well as combinations thereof contained in eye drops produced according to current pharmaceutical standards. Aqueous extracts from *Atropa belladonna* L., *Echinacea pallida* (Nutt.) Nutt., *Mercurialis perennis* L., *Euphrasia officinalis* L. and an aqueous dilution of *Rosae aetheroleum* were tested for their mutagenic potential in an *in vitro* bacterial reverse mutation assay following OECD standards. Extracts did not cause gene mutations and are thus considered to be non-mutagenic. To evaluate the eye irritation potential of eye drops containing combinations of the aqueous extracts listed above, an *in vitro* ocular irritation assay (OECD 492) using the EpiOcular™ human tissue model was conducted. Results classified the eye drops as “non-irritant” in accordance with UN GHS “No Category”. The applicability of both *in vitro* assays to more complex multicomponent mixtures is considered to be given, as no non-specific reactions with the test items were observed and all



criteria of validity were met. Therefore, this study should encourage the use of *in vitro* methods for safety assessment of herbal medicinal products.

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## Multicentric study of SkinEthic™ HCE time-to-toxicity method for serious eye damage/eye irritation assessment

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Regarding the assessment of eye toxicity, chemicals are classified according to the Globally Harmonised System (UN GHS) as Category 1 (serious damages), Category 2 (eye irritation) or No Category (not requiring classification). Considerable progress has been made towards replacement of Draize assay and several non-animal test methods have been validated and received regulatory approval. These *in vitro* methods are now available for incorporation into batteries of tests for new chemicals. Whilst test methods are capable of predicting Cat. 1 and/or No Cat., Cat. 2 predictions remain challenging. Therefore, SkinEthic™ HCE Time-to-Toxicity method, based on 2 protocols, one for liquids (TTL) and one for solids (TTS) was developed to differentiate between the whole range of classification.

A program of methodology transfer and assessment of within/ between laboratory (WLR/BLR) reproducibility was conducted involving multiple exposure time treatments (5 to 120-minutes). The relevance and reliability of both TTL and TTS have been assessed in a multi-laboratory trial involving 3 laboratories on 40 coded chemicals (20 liquids/20 solids), with 25 totally naïve chemicals according to OECD GD34 principles.

The WLR from the 3 laboratories was 90% for liquids and 100% for solids while BLR was 80% and 100% under UN GHS classification, respectively. Application to 40 chemicals, accuracy value was above 86%, with 90% Cat.1 (N = 14), 80% Cat.2 (N = 14) and 92% No Cat. (N = 12) correctly identified. Furthermore, with a sensitivity of 96% (28 Cat.1/2) and a specificity of 92% (12 No Cat.), the robustness of the method was confirmed. In conclusion, the SkinEthic™ HCE Time-to-Toxicity is an efficient transferable and reproducible method.

Therefore, an independent international peer-review panel concludes that the validation study and its conclusions are of suf-

ficient quality and completeness to allow an assessment of the scientific validity of the SkinEthic™ HCE TTT which is now under OECD program to receive regulatory approval.

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## Chemically defined cell culture media – A contribution to address the reproducibility crisis in biomedical sciences

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The use of fetal bovine serum (FBS) in cell culture media has different drawbacks. The harvesting of FBS from bovine fetuses after slaughter of the pregnant parent (dam) raises ethical and legal concerns. From a pure scientific point of view the use of FBS is unacceptable since regional differences in type and concentration of ingredients exist (Baker, 2016; van der Valk et al., 2010). Hence, a non-definable quality of FBS undermines data reliability and decrease or even prevent experimental reproducibility. However, the use of chemically defined cell culture media is still scarce.

The introduction of chemically defined cell culture media is a contribution to fight the reproducibility crisis in the biomedical sciences and an approach to address animal welfare concerns. Usage of chemically defined medium will eliminate some unknowns in cell culture experiments. A procedure to develop serum free media was introduced by van der Valk and colleagues (van der Valk et al., 2010). In the presented work, a detailed recipe to prepare a defined DMEM / Ham's F12 + ITS cell culture medium is given. This medium has proven to work in our laboratory for L929 and Caco-2 cell lines in combination with a certain plastic ware (Greiner Bio-One, Advanced-TC). To prepare the chemically defined cell culture medium (DME / F12 + ITS) mix 50% DMEM (e.g., Sigma Aldrich, D5648) and 50% Ham's F12 (e.g., Sigma Aldrich, N6760), add 14.7 mmol/l NaCl, 20.9 mmol/l NaCHO<sub>3</sub> and 5 ml/l ITS (Sigma Aldrich, I3146) (cellasys, 2019). The medium was developed for cell culture in a 5% CO<sub>2</sub> incubator. The presented method was employed to develop a chemically defined cell-based assay for cytotoxicity determination according to ISO10993-5 (Wiest, 2017), furnishing a first proof of its applicability as an alternative to cell culture media containing FBS.

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## Real life application of proposed guidelines for hiPSC banking in an academic environment

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Human induced pluripotent stem cells (hiPSC) are a promising tool to replace animal experiments, but only quality-controlled research material ensures the reproducibility of data. While there are publications available dealing with the need for global harmonization of quality standards for stem cell banking centres and commercial providers, or on the future pre-clinical and clinical use of the cells, up to date there are no standardized guidelines for the characterization and quality control of hiPSC in an academic research environment.

To fill this gap, we propose a two-step-banking process consisting of one Master Cell Bank (MCB) per hiPSC line, which is fully characterized regarding identity, cell antigen expression, mycoplasma contamination, karyotype, cell gene expression, pluripotency, cell count and viability, colony morphology, and post-thaw recovery, and respective Working Cell Banks (WCB), which undergo a partial characterization.

Here we present the results of the proposed testing panel of two hiPSC MCBs and show that both MCBs fulfil the quality criteria of (i) stem cell morphology, (ii) a normal STR profile, (iii) more than 50% viable cells, (iv) over 70% staining for each analyzed stem cell marker (FACS), (v) no mycoplasma infection, (vi) a normal karyotype, (vii) clustering with other hiPSCs in the PluriTest™, (viii) the ability to differentiate into cells of all three

germ layers, and of passing of the post-thaw recovery tests with (ix) > 70% living cells, (x) a stem cell morphology, and (xi) a confluency of > 70% after maximal 7 days in culture after thawing.

In conclusion, we propose a panel of eight assays, giving information on eleven quality criteria, which are practical and useful for an academic lab working with hiPSCs to ensure the quality of the cells at all times and provide the estimated costs for the whole banking process.

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## The human induced pluripotent stem cell (hiPSC) test as an alternative method for developmental toxicity testing

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Up to date the testing of chemicals for their potential to be developmental toxicants is mainly performed *in vivo* according to guidelines from the OECD or US EPA. As the testing of all registered chemicals *in vivo* is logistically and financially not feasible and bears the problem of species differences, there is an urgent need for alternative human-based methods. The mouse embryonic stem cell test (mEST), which assesses compound effects on cardiomyocyte differentiation from mouse embryonic stem cells, is an already existing alternative method validated by the European Center for Validation of Alternative Methods. However, the test is based on a mouse embryonic stem cell line and thus might not necessarily predict human toxicity.

To overcome this issue of species differences, we established the human induced pluripotent stem cell test, which is based on hiPSC. Similar to the *in vivo* situation during early embryonic development, the WNT and BMP signaling pathways are manipulated *in vitro* to obtain beating cardiomyocytes after 7 days in culture. Here we show the characterization of the cell model using (i) a cell viability assay (CTB), (ii) RT-PCR with markers of different developmental stages of cardiomyocytes, (iii) FACS and (iv) video analysis using 5-Fluorouracil (positive control) and Penicillin G (negative control) as model substances.

The hiPSC Test is a promising human *in vitro* assay for the identification of developmental toxicants. Predictivity of the methods needs to be assessed by testing positive and negative compounds in the future.

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## Optimizing the generation of human induced neural progenitor cell (hiNPC)-derived functional neuronal networks for use as alternative models in neurotoxicity testing

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Animal studies are the current standard for neurotoxicity (NT) testing, however, due to their high resource needs and low-test throughput, insufficient numbers of chemicals can be tested. The resulting knowledge gap and species differences between rodents and humans demand the development of alternative methods to assess the NT potential of chemicals faster, more cost-efficient, and with increased relevance for the human society. Here, we expand our method portfolio with neurally-induced human induced pluripotent stem cells (hiPSCs) for setting up test method that assess disturbance of neuronal network formation as well as acute neurotoxicity.

We compared three different 2D neural induction protocols for the generation of hiNPCs from hiPSCs and two media for the subsequent differentiation. All protocols featured dual inhibition of the SMAD signaling pathway, yet two of them were modifications of published protocols, while one was a commercially available kit (Stemcell Technologies™). After the 2D neural inductions, single cells were transformed into neurospheres and pre-differentiated by cultivation on a shaking platform. The emergence of functional neuronal networks was assessed upon plating the pre-differentiated neurospheres on microelectrode arrays (MEAs). The different cell models were characterized for stemness and neural marker expression at different time points using FACS, qPCR, and immunocytochemistry.

All induction protocols generated hiNPCs expressing the proliferation marker Ki-67 and the NPC marker Nestin and Pax6 while losing expression of the stem cell marker Oct 3/4. However, we observed distinct transcriptional signatures of specific brain regions, i.e., forebrain (FOXP1), midbrain (LMX1A), or midbrain (EN1)/hindbrain (HOXA2) depending on the applied protocol. Although all hiNPCs generated neuronal networks consisting of MAP2-positive neurons and S100β-positive astrocytes, differences in the neuronal subtype composition and synaptic marker expression were detectable. Moreover, assessment of the electrical activity revealed differences in the functionality of the neuronal networks depending on the induction protocol and the differentiation medium.

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## Identification and characterization of thyroid hormone disruptors in developing 3D neural progenitor cells *in vitro*

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Thyroid hormones (TH) play an important role during brain development as they are involved in multiple neurodevelopmental processes, e.g., myelin formation, necessary for oligodendrocyte (OL) function. The impact of environmental endocrine disrupting chemicals that interfere with the TH signaling pathway is a major concern with direct implications on children's health. Identification of TH disruptors is currently mainly performed as part of *in vivo* guideline studies for developmental and reproductive toxicity. However, these studies determine maternal TH serum levels only and neglect known TH-dependent neurodevelopmental effects in the offspring.

Therefore, there is an urgent need for an alternative *in vitro* method which is able to identify environmental TH disruptors in developing brain cells.

We developed an *in vitro* 3D neurosphere test method based on human and rat primary neural progenitor cells (NPCs), which allows assessment of TH-dependent OL maturation by quantifying expression of myelin-associated genes (hMBP, rMog) in relation to differentiated O4 + OL in presence or absence of TH and/or the chemical of interest (NPC6). Using this assay, we evaluated the adverse TH disrupting effects of Tetrabromobisphenyl A (TBBPA) and Perfluorooctanoic acid (PFOA) in comparison to the competitive TH receptor antagonist NH-3. Additionally, we performed microarray analyses for gaining deeper mechanistic understanding. Data was placed in an AOP-context.

TBBPA altered OL maturation, while PFOA was not effective. Human NPCs appear to be more sensitive than rat NPCs. We identified two modes-of-action how TBBPA interferes with the establishment of a population of maturing OLs, dependent and independent of TH signaling: (i) TBBPA as a TH disruptor impairing human OL maturation by dysregulation of oligodendrogenesis-associated genes. (ii) TBBPA disrupted genes regulating cholesterol homeostasis, reducing OL numbers independently of TH signaling.

The NPC6 assay is a promising alternative method to identify TH disrupting compounds in developing brain cells of human and rat origin.

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## Development and characterization of 3D spheroids for pre-clinical application

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A three-dimensional (3D) setup for the culture of mammalian cells is clinically more relevant as compared to monolayer culture as it can provide an environment that mimics cellular orientation and micro-architecture that exists *in vivo* (Fennema et al., 2013). Studies performed in 3D may reduce the number of candidates that may further be evaluated in animal investigations and clinical studies, thus providing an alternative to it. With the increasing use of nanoparticles in the field of medicine, their toxicity evaluation becomes important (Sambale et al., 2015). A simplistic 3D cell culture model of lung tumor for its potential in evaluating Eudragit (RL100) based nanoparticulate system has been studied here. A 3D mono and co-culture model was developed using epithelial and fibroblast cell lines. Ultra-low attachment surface allowed cell-cell adhesion resulting in aggregation and compaction of cells and subsequently development of spheroids. A549 cells formed spheroids by day 3 and showed an increase in diameter from 300  $\mu\text{m}$  to 400  $\mu\text{m}$  in 10 days. Use of cell tracker dyes confirmed the presence of both cell types within the co-culture spheroids. Quantification of nanoparticle uptake using flow cytometry after dissociating spheroids showed > 90% internalization. It was found that co-cultures were more resistant to drug loaded nanoparticles than monocultures. The findings demonstrated that lung co-culture spheroids were a suitable model for evaluating newer drug delivery systems.

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**Presentation:** Poster

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## Investigation of neurodevelopmental toxicity of Chinese herbal medicines using a 3D human primary neural progenitor cell assay *in vitro*

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Traditional Chinese Medicine (TCM) has been practiced for thousands of years to prevent disease. Especially Chinese Herbal Medicines (CHMs) have been widely used during pregnancy to promote health of mothers and fetuses. However, toxicities of most CHMs have not been thoroughly investigated. Considering the fact that the developing brain is a sensitive target, it is essential to investigate the developmental neurotoxicity (DNT) of CHMs. Currently, DNT testing is done according to an OECD or EPA *in vivo* guideline study, but these studies are insufficient for large scale DNT testing as they consume high amounts of animals, time and money and bear the issue of species extrapolation.

We have developed 3D neurosphere *in vitro* test methods (NPC1-5) based on human primary neural stem/progenitor cells (NPCs), which assess neurodevelopmental key events (KE), like NPC proliferation, migration and differentiation into neural effector cells (astrocytes, neurons and oligodendrocytes). Using this model, we analyzed adverse effects of the CHMs Tian Ma (TM) and Lei Gong Teng (LGT). Additionally, microarray analyses provide further insight into the modes-of-action of CHM toxicity. Data was placed in an AOP-context.

The classified non-toxic TM disturbs only differentiation into oligodendrocytes at the highest concentration in human NPCs, while it is negative for the same endpoint in rat NPCs. Based on transcriptomic changes, we hypothesized that oxidative stress might play a (major) role in the oligodendrocyte reduction. hNPC migration was the most sensitive endpoint (MSE) upon cellular LGT exposure and time-lapse microscopy of migrating NPC visualizes that LGT inhibits cell adhesion without affecting mo-



tility. Co-treatment with laminin indicates that LGT disturbs laminin-dependent cell adhesion, which is in accordance with our previously published AOP (Barenys et al., 2016).

The neurosphere assay is a valuable tool in compound hazard identification, including the compound class of CHM. For risk evaluation, exposures and IVIVE are needed.

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## Maturation of human induced pluripotent stem cell-derived cardiomyocytes by mechanical stimulus

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The human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CMs) provides the source of human CMs for cell therapy, drug testing, and disease modeling. However, the function and structure of hiPSC-CMs are similar to immature fetal CMs than mature adult CMs. There are many successful methods that have been developed for the maturation of hiPSC-CMs, such as long-term cultures for > 100 days, electrical and/or mechanical stimulation, co-culture with non-CMs, etc. Because CMs evolve in a mechanically active environments generated by spontaneous contraction, the exploring mechanical cue for the maturation of hiPSC-CMs may thus suggest a strong biomimicking rationale for producing functional (or spontaneously beating) myocardial tissue. Therefore, this study aims to elucidate the effects of mechanical stress on the maturation of hiPSC-CMs and its underlying mechanism. In this study, we found that cyclic stretching induced the electric maturation of hiPSC-CMs, and the increased cell size, and the decreased circularity. Cyclic stretching also increases the ion channel expression, which is important to cardiac action potentials and function. In addition, the RNAseq analysis demonstrated several key molecules related to the maturation of hiPSC-CMs. These results provide a new insight to the relationship of ion channel and maturation-related molecules during the physical stimulation of iPSC-CMs.

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**Presentation:** Poster

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## Foster and fund: Enhancing 3R activities at Charité – Universitätsmedizin Berlin by Charité 3R

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Charité, the university hospital in Berlin, is the joint medical faculty of the Freie Universität Berlin and the Humboldt-Universität zu Berlin and conducts about 50,000 animal experiments per year for biomedical research. Since 2018, it actively strengthens the in-house implementation of the 3Rs by the foundation of Charité 3R (<https://charite3r.charite.de/en/>). The faculty body supports 3R research and education by research funding, workshops and courses. The measures are accompanied by communication and outreach activities.

Up to now, 7 calls for 3R research projects have been published. For these, 88 proposals have been submitted and evaluated by independent peer-reviews. As a result, 33 projects have been funded with a total of 3.7€ Mio. The calls focus on refinement, innovative high risk research ideas and other 3R approaches that are rarely funded by public funding agencies. Charité 3R aims to incentivize the change towards alternative methods and draw the attention to the relevance of refinement and reduction for high quality biomedical research results. To disseminate 3R knowledge within young researchers, Charité 3R provides 3R courses for PhD students. In addition, Design Thinking Workshops for the creation of new 3R approaches are organized for PhD students and postdocs (“Re-Think3R”). Outreach activities focus on public events, transparent communication on 3R research and collaborative strategies with the animal welfare officers.

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## Cytotoxicity of ZnO micro- and nanoparticles on HaCaT and A549 studied by MTT, NRU and LDH viability assays

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Nanomaterials are one of the most versatile products but due to their intrinsic physicochemical properties they can interact with various biological molecules or cells causing alterations in the organism. Consequently, the purpose of this work is to evaluate the potential cytotoxic effect of ZnO micro and nano particles on two human cell lines, HaCaT and A549, using three different endpoints, namely MTT, NRU and LDH. This comparative *in vitro* cytotoxic study will increase the knowledge on how metal nanoparticles develop adverse effects.

The mean hydrodynamic diameter and the polydispersity index (PDI) of the particles were determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). Before measurement, the particles were appropriately diluted in phosphate buffered saline (PBS, pH 7.4) and DMEM 5% FBS, and incubated for 2 h and 24 h at room temperature, at a final concentration of 1.0 mg/ml. We follow the protocol of Vinardell et al. (2017) for cell culture and treatment. After 24 h of treatments, cytotoxicity was assessed by the MTT and NRU viability assays and the LDH test.

Data from hydrodynamic diameter indicate that this variable increases with time. This fact maybe due to agglomeration phenomena or to possible adsorption of proteins on the particle surface, to a greater or lesser degree (Allouni et al., 2015). In the case of cytotoxicity, we have calculated IC50 for better comparisons among the different products and cell lines. IC50 is in general inferior to 100 µg/ml IC50 except for the case of NRU, because cell viability at the maximum concentration assayed is approximately 80%. In general, comparing the different zinc oxides, micrometric ZnO is the most cytotoxic, followed by 100 nm ZnO and 50 nm.

In general, we can conclude that a relationship between nanometric size and higher toxicity cannot be directly attributed.

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## Self-organized pigmented and vascularized full skin tissue model for melanoma research

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Being a multi-layered organ, the complexity of human skin is difficult to mimic *in vitro*. Despite the major advances in currently available 3D skin models, they remain very simplistic in relation to native tissue complexity. Moreover, the integration of melanoma cells into 3D skin models rendering representative tumor microenvironments is yet to be fully achieved. Cell sheet engineering has shown impressive results in recreating the native tissue structure and interactions of many different tissues by keeping intact the native cell-cell and cell-extracellular matrix interactions (Yanf et al., 2007; Haraguchi et al., 2012). Considering these major features, we built a 3D full skin model by stacking heterotypic cell sheets of human dermal fibroblasts (hDFBs) and keratinocytes (hKCs) or of hDFBs and human dermal microvascular endothelial cells (hDMECs), respectively as units of the epidermis- or dermis-like analogues. Melanocytes were further incorporated in the epidermis-like cell sheets before exposed to air-liquid interface (ALI), obtaining a pigmented stratified epidermis. In order to understand how melanoma cells responded to the self-organized full skin model, different cell lines (WM-115, SK-Mel-28, LM-MEL-33 and VMM15) were used. Histologically analysis confirmed the organization of those cells within the epidermis and their migration into the dermis through the formed basement membrane. Interactions between the melanoma cells and the stromal cells in the dermis are being assessed focusing on the known mechanisms involved in tumour progression in order to validate our approach.

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## Differentiation of motor neurons for *in vitro* potency estimation of Botulinum neurotoxins

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Botulinum neurotoxins (BoNTs) are bacterial toxins that cleave SNARE proteins, which are essential for neurotransmitter release in neurons. This results in a flaccid paralysis through inhibition of motor neurons (MNs). The serotypes BoNT/A1 and B1 are used for medical and aesthetic applications, for which the potency estimation of every produced batch is a prerequisite. Annually, 600,000 mice are used worldwide, despite the fact that cell-based alternative methods are available. However, these alternatives are specific for unique BoNT serotypes. As new BoNT products are expected to enter the market, a universally applicable *in vitro* assay for all BoNT serotypes would be advantageous. Since there are differences in sensitivity to BoNTs between humans and mice, human MNs are the most physiologically relevant target cell type with presumably high sensitivity. Therefore, MNs were generated from human induced pluripotent stem cells *in vitro* with three optimized differentiation protocols. The resulting neuronal cell populations were analyzed for their suitability to be used in the potency estimation of BoNTs in comparison to the neuroblastoma cell line SiMa, which is commonly used for BoNT/A potency estimation. For this, the cleavage of the respective substrates was quantified via Western Blot. Interestingly, human MNs not only showed a much higher sensitivity to BoNT/A1 than SiMa cells, but also high accordance to data from mouse lethality assays. MNs were less sensitive to BoNT/B1, which is in line with species differences in the sensitivity for this serotype, while SiMa cells did not show a dose-related response. To conclude, MNs can be used for potency estimation of BoNT/A1 and B1 and are a physiologically relevant and sensitive model, which could vastly improve current methods for estimation of BoNT activity.

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## Implementing the principles of 3Rs in the project application, evaluation, and authorization process in the University of Debrecen Committee of Animal Welfare's work

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The tasks of Animal Welfare Bodies are defined by the European Union's (Directive 2010/63/EU of the European Parliament and of the Council; Article 26 and 27) and national legislations (in Hungary the Decree 40/2013 of the Hungarian Government; Article 39). One of the duties of Animal Welfare Bodies' is the supervising of the authorization of scientific projects at the institutional level. The Animal Welfare Body of the University of Debrecen (named Committee of Animal Welfare) is participating actively during the application process for scientific projects.

To acquaint the researchers with the project application procedure, at the University of Debrecen in the Laboratory Animal Science and Welfare Courses' was introduced new topics as follows: in Function Specific (Prerequisite) Modules the "Design of procedures and projects (Level 1 and 2)" as lectures and in the Additional Tasks Specific Modules the "Project evaluation" as seminars. These topics can help the participants to familiarize themselves with current legislation, with the application form, and the project application procedures. Moreover, the researchers can learn how to design a project, how to apply the principles of 3R's during animal experiments, how to approach the severity assessment, and how to perform the retrospective analysis of the experiment.

We would like to present with examples and statistical data on how the EU/63/2010 Directive changes the attitude of the researchers to apply the objectives of animal welfare during their scientific work.

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## Multimodal monitoring in a preclinical study: Wheel running behavior uncovers impaired welfare due to serial intraperitoneal injections

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In many research fields, experiments still substantially rely on animal models. Routine procedures such as frequent handling and injections are performed according to the research aims with little consideration of the impact on the animals, despite the influence on study outcome and data quality. Therefore, this study monitored a preclinical experiment by assessing voluntary wheel running as a measure of general well-being. The study was hereby aiming to identify impacts potentially being missed by conventionally applied methods to enable refinement of the procedures in future.

The study comprised a laparotomy for pancreatic cancer cell injection and a subsequent phase with daily intraperitoneal injections of chemotherapy and vehicle substances. After laparotomy, minimal body weight reductions, slightly elevated clinical scores but significantly decreased voluntary wheel running (VWR) behavior were observed. Following the repeated intraperitoneal injections, body weight was reduced in response to the chemotherapy, but not after vehicle treatment, while the activity-related behavior was decreased in both cases. Wheel running behavior further revealed differences between the substances and altered nightly activity patterns.

By assessing voluntary wheel running, this study demonstrated a high impact of the repeated injections and differences between substance effects on well-being. Moreover, it revealed VWR as a more sensitive indicator of impairment, strongly suggesting the need for multimodal severity assessment and refinement of experimental protocols. However, differences in tumor growth between the treatment groups could not be determined. This might be due to the high impact of the procedures uncovered in this study, as exaggerated stress responses are potentially confounding factors in preclinical studies.

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## Recommendations on group housing of male mice – A compilation of experiences from Swedish laboratory animal facilities and scientific literature

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Animals used for scientific purposes are protected by EU legislation. Social animals should be kept in stable groups that enable species-typical social behavior and provide individuals with social comfort. However, when group housing male mice, aggression within the home-cage is a common husbandry and welfare problem. Excessive fighting and injuries due to aggression can cause pain and stress, resulting in individuals being euthanized or housed individually. In addition, stress can alter physiological parameters, risking scientific validity and generating larger sample sizes.

Mouse aggression, and the consequences thereof, thus opposes the 3R goals of Refining the methods to minimize potential pain and suffering and Reducing the number of animals used within research. Therefore, the aim of this project was to gather information on strategies used in Sweden when group housing male mice, to compare this information to literature collected through a systematic search strategy, and finally, to produce recommendations on how to prevent aggressive behavior.

During 2018-2020, we have worked with external experts to collect a broad knowledge base from Swedish laboratory animal facilities as well as scientific literature (Zidar et al., 2019). Based on the collected data we have compiled recommendations aimed at preventing aggression in group housed male mice and thus improve animal welfare, the working environment and, consequently, the quality of research.

The recommendations cover the practicalities of animal housing and the overall work method – procedures, cooperation, and communication. As many of these aspects have not been sufficiently studied in scientific literature, staff experiences are a crucial source of knowledge.



The recommendations are available in English and Swedish at our webpage: <https://jordbruksverket.se/3R>

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### Development of a statistical prediction model for skin sensitization by combining multiple *in vitro* tests

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Development of skin sensitization evaluation system without animal testing is one of the major issues in cosmetic safety assessment. It is considered that single *in vitro* test may not replace fully conventional animal testing, because skin sensitization is complex immune systemic reactions (OECD, 2019). Thus, it is important to evaluate by a combination of multiple tests following AOP (Adverse Outcomes Pathway), and IATA (Integrated approach to testing and assessment) included them is being studied worldwide. Several case studies of IATA are introduced in OECD's Guidance Document for skin sensitization (OECD, 2016). However, the majority is to use expensive *in silico* models or machine learning systems that lack transparency.

In this study, we developed prediction models that predict skin sensitization categories determined from EC3 in LLNA (local lymph node assay) by combining *in vitro* tests following skin sensitization AOP. We selected SH-test (Susuki et al., 2009) (protein binding), KeratinoSens™ (keratinocyte activation), and h-CLAT (dendritic cell activation) as *in vitro* skin sensitization tests. To construct a new prediction model, we examined two statistical models, a liner regression model and a proportional odds model with the published data of 136 chemicals including the results of three *in vitro* tests and LLNA (Urbisch et al., 2015).

The predictive capacity of the liner regression was poor performance. The reason is considered that any constant value of EC3 for Non-sensitizer in LLNA was substituted. On the other hand, the proportional odds model showed high concordance rate.

We propose the developed predictive model using the proportional odds model. The model is able to develop a relatively simple method with excellent robustness and transparency.

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### Marching towards Asian Federation for Alternatives to Animal Testing (AFAAT) through harmonization of Asian 3R centres and associations for alternatives

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In as much as West European and North American countries forge ahead in discovering, validating and practicing alternative methods the situation in developing countries, especially Asia, is not anywhere near. Adoption of alternative methods discovered and validated elsewhere and contribution to discovery of newer methods for international harmonization by these countries can be greatly motivated and encouraged through establishment of national societies. In Asia, Japan, through its JSAAE, stands a unique exemption. 3R/Alternatives Societies have come up in South Korea, China, India and Sri Lanka but national priorities being different the alternatives scenario is not encouraging. The JSSAE has taken the initiative of a federation of these societies. Nevertheless, international harmonization at the global level such that the countries which are less endowed are pushed forward by those that are better endowed, will be rewarding.

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## A human iPSC-based *in vitro* neuronal network formation assay to investigate neurodevelopmental toxicity of pesticides

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The spatiotemporal orchestration of key neurodevelopmental processes (KNDP) is essential for brain development. An adverse outcome, i.e., developmental neurotoxicity (DNT), is expected, if at least one KNDP is affected due to exposure towards a compound during a critical period of neurodevelopment. The ultimate, functional readout for nervous system function *in vitro* is the formation and function of neuronal networks assessed via the neuronal network formation (NNF) assay.

The human NNF assay is based on network development of hiPSC-derived human GABAergic inhibitory and glutamatergic excitatory neurons, as well as primary human astroglia (NeuCyte, USA). These pre-differentiated cells are seeded in a standardized ratio on 48-well microelectrode array (MEA) plates. After one week of differentiation in absence of the test compounds, network activity is measured via the assessment of spike-related, burst- and network-related parameters using the Axion Maestro Pro System. These measurements serve as internal baselines for subsequent developmental exposure and later readouts from day 7 to day 35 *in vitro*. Currently, a set of 28 pesticides, that are known to either affect or not affect brain development based on the rodent DNT guideline study (OECD TG426), is tested in the NNF assay.

Results show that some pesticides like deltamethrin cause concentration-dependent effects on the mean firing rate and the synchronicity of the network. Other compounds like flufenacet were identified as negative.

In a next step the effects of the 28 pesticides on the NNF assay will be compared to results from assays that covers other KNDP like proliferation, migration, neuronal differentiation and oligodendrocyte formation as well as the rat NNF assay. The human NNF assay is a valuable addition to the current DNT *in vitro* testing battery as it converges on multiple neurodevelopmental key events like neurite outgrowth, dendritic spine formation and synaptogenesis.

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## The cardiac embryonic stem cell test shows presence of biomarkers for endodermal, ectodermal, and neural differentiation

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A well-studied screening tool for developmental toxicants is the cardiac embryonic stem cell test (ESTc). This *in vitro* test makes use of embryonic stem cells (ESCs) followed by embryoid body (EB) formation and spontaneous differentiation towards cardiomyocytes. Also, other cell types differentiate within these EBs and could potentially be of additional use to enlarge the applicability domain of the test. Therefore, this study aimed to further investigate the biological domain of the ESTc by studying the presence of additional cell types. Several biomarkers were investigated in time by performing immunocytochemistry and gene expression analysis (RT-qPCR). Proteins were present for endodermal cells (AFP), neuroectodermal cells (NES), and neural cells (TUBB3) within the ESTc in addition to cardiomyocyte differentiation. Endodermal differentiation (AFP) over time, showed clearest results in the early differentiation track and diminished on differentiation day 10. Neuroectoderm (NES) and neural (TUBB3) differentiation emerged on differentiation day 4 of the ESTc. NES and TUBB3 positive cells were especially located at the EB borders. In time, the composition of TUBB3 versus NES positive cells changed, with a higher presence of TUBB3 positive cells on differentiation day 10. Additionally, TUBB3 staining also showed axon-like structures from differentiation day 4 onwards. The tested markers showed upregulations on gene expression levels over time. Comparing immunocytochemistry results and gene expression levels in case of TUBB3, showed protein composition changes although gene expression levels didn't show clear upregulations in time. This difference in sensitivity between these biomarkers should be considered when selecting gene transcript biomarkers for toxicity screening purposes. Taken together, these results show further application of the ESTc through enlarging its biological applicability domain. This is promising when it comes to expanding the applicability domain of the ESTc for toxicological screening purposes in the future.

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## In vitro tests of stone wool fibres dissolution

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Stone wool fibres are widely applied in the construction industry for heat insulation and fire spread protection. Potential long term health effect of fibre materials upon inhalation by humans is studied by biosolubility *in vitro* and biopersistence *in vivo* investigations. A reliable, robust and reproducible *in vitro* test procedure is required to build a correlation between *in vitro* and *in vivo* tests which is necessary in the wish to reduce animal use for *in vivo* tests.

Here we report an *in vitro* dissolution test procedure using a flow-through reactor setup with low dead volume for determination of a fibre dissolution rate. The fibre dissolution rate was measured for fibre samples with the same initial mass under constant temperature (37°C) and flow rate (125 ml/day) of a modified Gamble's solution (initial pH 4.5) over 21 days to simulate intracellular macrophage phagolysosome conditions. The intended flow-to-surface area ratio (F/A) was equal to 0.030 µm/s. The dissolution rate was estimated by ICP-OES measurements of the number of elements released from the fibres to the effluent solution. Element concentrations were normalized using sample surface area calculated from fibre diameter distribution obtained using scanning electron microscopy (SEM).

It was shown that deviations from set flow rate and pH increase caused by dissolution reaction significantly influence the dissolution rate. Increase of the solution pH decreases the dissolution rate, while changes of flow rate influence reproducibility of the results. Reproducible determination of the fibre sample surface area and its decrease caused by dissolution reaction are essential for F/A ratio settings in the experiments and dissolution rates calculations. It was concluded that it is important to keep F/A constant to obtain reproducible results and increase of pH the modified Gamble's solution can be avoided by introducing buffers at pH 4.5.

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## Normothermic machine perfusion of human diseased ex vivo livers to study hepatic pharmacokinetic processes

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The prediction of hepatic clearance and biliary excretion is of high importance to assess the pharmacokinetics of drugs, especially in patients suffering from hepatic diseases with altered liver function. We therefore aim to develop a physiologically relevant human pre-clinical *ex-vivo* model to investigate hepatic clearance, biliary excretion by utilizing normothermic machine perfusion (NMP) of diseased human livers.

Patients waitlisted for liver transplantation were given the opportunity to participate in this study. During liver transplantation procedure, the portal vein and the hepatic artery of the explanted liver was immediately flushed, and reconstruction of the portal vein and hepatic artery was performed. Perfusion was initiated using the LiverAssist device at a temperature of 37°C for 360 min. After 120 min, a bolus of a drug cocktail (rosuvastatin, digoxin, furosemide and metformin) was applied and at t = 240 min, again a bolus of the cocktail was co-administered with inhibitors.

Seven explanted livers of patients undergoing liver transplantation were included in this study: Primary Biliary Cirrhosis (PBC), Non-alcoholic Steatohepatitis (NASH), 3x Alcoholic Liver Disease (ALD), and 2x Hepatocellular carcinoma + Hepatitis B viral disease (HBV+HCC). All livers except PBC produced > 30 mL bile throughout the duration of the experiment. A relation was shown between the severity of the underlying disease of the patient and functioning of the liver during perfusion (lactate levels, AST, ALT). The non-cirrhotic livers showed to rapidly clear the drugs from the perfusate in contrast to the cirrhotic livers which showed a delayed plasma clearance. Differences in biliary clearance were observed which are related to the transporter expression of the underlying disease.

Here we demonstrate for the first time that it is feasible to perfuse explanted diseased human livers to study hepatic clearance, biliary excretion under specific disease circumstances. We will further explore the potential of this model by studying other drugs and drug-interactions in comparison to clinical data

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## Alternative method to culture intestinal organoids without loss of biological functionality

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Over the last years, organoids have become increasingly popular in research as they resemble the composition and functionality of organs, making the organoid model an ideal alternative to *in vivo* testing. Intestinal organoids are derived from freshly isolated intestinal epithelial crypts that harbor Lgr5-expressing stem cells. A method for long-term culture of 3D murine intestinal organoids was first developed by Sato et al. Several essential factors to maintain basic crypt physiology were described: Wnt/R-spondin1 to activate the Wnt signaling pathway, Noggin as a BMP4 inhibitor and EGF to stimulate proliferation. For structural support and biochemical cues, organoids are cultured in Matrigel matrix.

In search for reduction of time and costs in culturing intestinal organoids, we screened several factors to replace one or more of the components used by Sato et al. This screening has led to the composition of a new culture medium. We developed and thoroughly tested our new culture medium and designed a standard operating procedure (SOP) for long-term maintenance of intestinal organoids. To standardize our method, the SOP was performed in three separate laboratories, in which a vial of murine ileum organoids from liquid nitrogen was thawed, expanded and maintained for 3 weeks, with weekly passaging. The viability of the organoids was assessed by observing morphology. In addition, the expression of IL22-induced gene expression of the anti-microbial peptide Reg3G was determined by exposing organoids to IL22 for 24 hours each week, followed by RNA isolation.

Results show that three laboratories were able to culture viable and healthy murine intestinal organoids with the newly developed culture medium and SOP. As expected, IL22 stimulation consistently induced Reg3G expression in the organoids. These results indicate that the developed SOP to culture murine intestinal organoids can be transferred to independent laboratories and can be used for long-term culture of intestinal organoids.

**Presentation:** Poster

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## Introducing Brazilian students to lobbying – Enacting legislation against cosmetic testing on animals

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Half a million animals are used on annually for cosmetic testing, despite the existence of validated non-animal tests. To address this problem, HSI launched the Be Cruelty Free (BCF) campaign in several countries. In 2012, BCF was launched in Brazil and despite many advances, a complete ban has not yet been achieved. The bill has moved through the two houses of the Brazilian parliament but has yet to become federal law – despite 10 states enacting a ban already. Over 70% of the Brazilian public wants robust federal legislation against cosmetic testing on animals.

In order to revitalize the bill, HSI have initiated a project with students between 14 to 17 years old, from three different schools in the capital Brasilia. The aims of this project were to engage with the next generation, to introduce students to the issues of animal testing and to facilitate their transition to effective lobbyists. For the first part of the project, students and teachers attended to lectures on animal testing provided by HSI explaining the need for a ban on cosmetic testing and the non-animal methods available to test cosmetics. The second step was to visit politicians, with students participating in a debate inside the Brazilian Parliament.

Our project revealed that the majority of students had not known about the use of animal for testing cosmetics, but after attending this program, they were unanimously against the practice. The politicians recognized this strength of opinion and committed to prioritize discussion of the bill at the plenary of the Senate.

We aim to continue this annually – continuing to engage young lobbyists and new politicians. Here, we also present strategies for expanding this program to enable other countries to use these approaches in response to the interests and concerns of their youth.

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## Retrospective analysis of dermal triple pack data

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Dermal toxicity is driven by the ability of a substance to penetrate the skin. Dermal absorption can be estimated using the “triple pack”, a study design that combines *in vivo* rat, *in vitro* rat, and *in vitro* human data to calculate an estimated human dermal absorption factor (DAF). To assess the feasibility of deriving a DAF using only *in vitro* data, we conducted a retrospective evaluation of agrochemical formulations to compare the DAF derived from each method. We also compared the DAF derived from the human *in vitro* study to the DAF generated from the triple pack approach. For most of the formulations evaluated, the *in vitro* rat method generated a similar or higher DAF value than the *in vivo* method. Absorption through *in vitro* human skin was found to be similar to or less than that observed in rat skin for all formulations. For most of the formulations, the human *in vitro* method provided a similar or higher estimate of dermal absorption than the triple pack approach. For human health risk assessment, *in vitro* assays using human skin would be preferable. Such tests would be directly relevant to the species of interest (which is humans) and avoid any overestimation of dermal absorption using rat models. However, rat *in vitro* studies would still have utility if human *in vitro* data were not available. *In vitro* rat data provide estimates of dermal absorption that are at least as protective as *in vivo* rat data, and thus could also be considered adequate for use in establishing dermal absorption factors. Accordingly, the comparisons presented in this poster support potentially using *in vitro* data alone for DAF derivation for human health risk assessment of pesticides.

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## Mass spectrometry-based quantification of all antigens in diphtheria-tetanus-acellular pertussis combination vaccines containing aluminum hydroxide as adjuvant

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Animal tests are, unfortunately, still common place in safety testing and vaccine batch release. Combination vaccines, such as DTaP (containing diphtheria toxoid, tetanus toxoid, pertussis toxoid, filamentous hemagglutinin, pertactin, and fimbriae 2 and 3) undergo strict batch release tests including *in vivo* assays based on serology and sometimes even challenge tests carried out in guinea pigs and mice. A single method in which accurate identification and quantitation of the antigens present in DTaP combination vaccines containing alum based adjuvant has remained elusive. As such, we have developed an LC-MS method that allows for both identification and quantitation of all antigens in DTaP-vaccines within a single analysis. The quantitation approach is based on isotopically labeled, antigen-specific signature peptides. The method can be used to identify and quantitate pre-adjuvanted monovalent antigens that have been chemically inactivated with formaldehyde. Even more, Al(OH)<sub>3</sub>-containing final vaccine lots can also be subjected to the method, thereby allowing for in-process and final product assessment. In addition, the specificity of the developed method was demonstrated using vaccines in which a single antigen has been intentionally omitted in the formulation. In summary, the LC-MS method ultimately paves the way to a reduction in the number of animals tests required for vaccine batch release.

*Acknowledgment: This method is developed as part of a collaborative research project called VAC2VAC. The VAC2VAC project is sponsored by the Innovative Medicines Initiative (IMI2); a public-private partnership between the European Commission and the Pharmaceutical Industries in Europe (VAC2VAC grant no 115924).*

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## Combinatorial model organism strategy to predict developmental and reproductive toxicity (DART)

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Many petroleum substances (UVCBs) have a production volume of > 100 tons/year. According to European legislation (REACH), they therefore need to be tested for prenatal toxicity. As this requires a huge number of test animals, innovative toxicity test systems that can be used during the R&D phase will be of clear added value in safety assessment. In the EFRO TOXFLOW project we aim to develop an innovative toolkit to predict DART of UVCBs. To this end we selected two organisms, i.e., the nematode *Caenorhabditis elegans* and zebrafish (*Danio rerio*) embryos, as first tier assays, and well-known cell-based assays like EST, CALUX and steroidogenesis assays, as second tier.

Here *C. elegans* results are presented. We optimized exposure methods to improve exposure-controlled hazard prediction of substances with different physicochemical properties. We confirmed the importance of these methods for different classes of chemicals. We also developed automated scoring methods to identify developmental and reproductive toxicity, as well as methods to assess neurotoxicity, chromosomal instability, and lipid levels.

Testing of > 50 chemicals confirmed the added value of both the exposure optimization and the sensitivity and predictivity of the *C. elegans* test system for different biological end points. This indicates the value of *C. elegans* as a first-tier test method.

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## Ultrastructural and functional characterization of a reconstructed human corneal epithelium (HCE) as an alternative to animal use

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The need for models of human corneal epithelia in pharmacology and toxicology fields leads to the development of three-dimensional (3D) corneal models. SkinEthic HCE is a reconstructed human corneal epithelia model produced by EPISKIN from cultivation of transformed human corneal epithelial cells on a polycarbonate membrane at the air-liquid interface in a chemically defined medium. This 3D construct resembles the stratified cellular organization of human corneal epithelium: a monolayer of columnar basal cells covered by intermediate cells similar to *in vivo* corneal wing cells and a non-keratinized superficial layer of flattened cells. Transmission electronic microscopy (TEM) imaging showed in all layers a well-developed cytoskeletal network and cells-cells connection with numerous desmosomes and, in the cells of the basal layer, anchoring hemi-desmosomes connecting the construct to the support. Scanning electron microscopy (SEM) surface analysis of SkinEthic HCE revealed structures resembling to the microvilli observed *in vivo*. These structures appear to be coated with a sort of mucus forming large plaques suggesting presence of a superficial glycocalyx layer as observed on human cornea. RT-qPCR analysis for mucin genes MUC1, MUC4, MUC5B, MUC5C and MUC16 on SkinEthic HCE confirmed expression of these genes excepted for MUC4. Immunohistochemistry of the membrane-anchored mucin, MUC1, showed a suprabasal location compatible with a glycocalyx-like accumulation. Cells of the suprabasal layer express also keratin 3, a human cornea-specific differentiation marker. Barrier function of the SkinEthic HCE model can be measured by ET50 and the observation by immunohistochemistry of occludin protein in all layers is in agreement with existence of dense tight junction network.

This work has showed some parameters similarities between SkinEthic HCE model and the *in vivo* situation in human. It confirms suitability of 3D construct to model human corneal tissue as an alternative to animal use.

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## Mixture-based QSAR models of ocular toxicity for regulatory hazard categories

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Computational modeling can be used to design effective non-animal approaches, if grounded in reliable experimental data. We have developed a set of computational models to predict eye irritation and corrosion. The models were developed using a curated database of *in vivo* eye irritation studies from the scientific literature and stakeholder-provided data. The database contains over 500 unique substances, including many mixtures, tested at different concentrations. Substances were categorized according to Globally Harmonized System (GHS) and U.S. Environmental Protection Agency (EPA) hazard classifications. Two modeling approaches were used to predict classification of mixtures. A conventional approach generated predictions based on the chemical structure of the most prominent component of the mixture. A mixture-based approach used weighted feature averaging to consider all known components in the mixture. Ranking accuracy rates (calculated based on the area under the receiver operating curve) for EPA hazard classification of undiluted test substances were 74-81% and 75-80% for the conventional and mixture-based models, respectively. Ranking accuracy rates for EPA hazard classification of substances diluted to 10% in the conventional and mixture-based models were 90-95% and 92-96%, respectively. Ranking accuracy rates for GHS hazard classifications for undiluted test substances were 79-82% and 80-91% for the conventional and mixture-based models, respectively. Rates ranged from 89-95% for the diluted GHS classification predictions for both approaches. We observed a strong correlation between a substance's pH and activity. Our results suggest that these models are useful for screening compounds for eye irritation potential. Future efforts to increase the models' utility will focus on expanding their applicability domains and using them in

conjunction with other input variables (e.g., *in vitro* data) to establish defined approaches for eye irritation testing.

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## Rat acute systemic toxicity testing: Evaluating reproducibility and inherent variability

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Regulatory agencies rely upon rodent *in vivo* acute oral lethality data to determine hazard categorization, assign appropriate precautionary labeling, and perform quantitative risk assessments. As the field of toxicology moves towards animal-free new approach methodologies (NAMs), there is a pressing need to develop reliable and robust reference data sets to contextualize results, to set expectations regarding NAM performance, and for training and evaluating computational models. To meet these needs, rat acute oral LD50 (dose corresponding to 50% lethality) data from multiple databases were compiled and curated. These data were analyzed to characterize variability and reproducibility of results across a set of more than 2400 chemicals with multiple independent study records. We did not have sufficient study metadata to evaluate the impact of specific protocol components such as species/strain, age, sex of rat, feed used, treatment vehicle, etc. However, we assumed studies had followed standard test guidelines, and thus evaluated several chemical-based possible sources of variability, including chemical structure, physicochemical properties, and functional use. We could not attribute the observed variability to any chemical-specific characteristics, and thus concluded that inherent biological or protocol variability is likely underlying the variance in the results. By bootstrapping across computed chemical-specific standard deviations, quantified variability was used to define a 95% confidence interval of  $\pm 0.25 \log_{10}(\text{mg/kg})$ . This confidence interval was used to define the uncertainty associated with discrete *in vivo* rat acute oral LD50 values and may serve as a benchmark to apply to future NAM performance assessments.



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## Whole blood thrombin generation test to reduce animals used for plasma collection and single dose studies

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Coagulation is a process by which blood clots are formed to stop bleeding from damaged blood vessels. For patients with hemophilia or other clotting disorders, monitoring of coagulation in blood is important for optimal treatment. Thrombin is an enzyme in the coagulation cascade which is essential for clot formation. Hence, thrombin generation in samples is an *in vitro* measure of coagulation potential and is commonly applied in mouse studies (as well as in clinical studies).

*In vivo*, other blood components and particularly platelets are important for coagulation, why it is often necessary to add platelets to the plasma prior to evaluation. However, whole blood would be even more physiologically relevant.

Existing procedure for evaluating procoagulant compounds requiring platelets has been *in vitro* thrombin generation tests (TGT) in platelet-rich-plasma (PRP-TGT), followed by a confirmatory single dose *in vivo* study. Using these methods, approximately 12 mice are required for each compound tested.

We replace the above methods with Whole-blood TGT (WB-TGT) in order to use fewer mice and to replace confirmatory *in vivo* studies with *in vitro* Dose/Response studies.

WB-TGT can be performed in about 1/4 of the volume of blood needed to produce plasma for PRP-TGT, thus saving mice. Furthermore, due to the increased translational value, *in vivo* Dose/Response studies can be designed without need for single dose *in vivo* studies, omission of which saves further mice.

This method has potential relevance in all research fields where coagulation is involved.

In conclusion, this reduces animal use since the same (or superior) results are achieved using fewer mice. Furthermore, there is an element of replacement since the results from this *in vitro/ex vivo* method can replace certain *in vivo* studies.

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## Development of an intestinal organoid-based platform for screening antiviral agents

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Enteric viruses are a major source of human diseases, particularly in neonates and young children. Despite being a pressing medical problem, very little is known regarding the events associated with the infection with these viruses on the human gastrointestinal (GI) tract. The interaction of such pathogens with the intestinal barrier depends on host-specific mechanisms that affect the success of an active viral infection *in vitro*. The use of immortalized cell lines, typically limited by the presence of only one cell type, have hampered our understanding of the host/pathogen interactions and the development of novel interventions against the infection with such pathogens. In addition, gastrointestinal toxicity is a major side effect of many drugs that limits compliance and thus limits efficacy of potential new drugs. Thus, a more reliable *in vitro* model is urgently needed to also reduce the work with animal models. Here, we aim to use primary stem cell-derived intestinal organoids to establish a new platform for antiviral drug discovery and as a pre-clinical tool to analyze drug toxicity. Intestinal organoids not only retain the genetic background of the patients from which they are derived, but also the differentiation of the adult stem cells can be steered towards all the major cell types that populate the GI epithelium, allowing to recapitulate the viral infection *in vitro* and better predict drug responses.

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## Fully human skin-on-a-chip with a modular architecture and integrated sensors for drug screening and disease modelling

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*In vitro* human skin models have been widely used as alternatives to animal testing for basic research and clinical applications. However, current commercially available models do not fully reproduce the structure and function of human skin, mainly due to their use of animal-derived collagen and their lack of a dynamic flow system to mimic vascularization.

In this study, we present an innovative, fully human skin-on-a-chip system that reproduces key aspects of the *in vivo* cellular microenvironment. Our approach combines the production of a fibroblast derived matrix with the use of an inert porous scaffold for long-term, stable cultivation of a human skin model, without the use of animal components. This advanced skin model is developed inside a biomimetic organ-on-a-chip system with a dynamic perfusion arrangement for continuous supply of nutrients and metabolites. The chip is reversibly sealed and has a modular architecture providing an easy-to-use workflow, precise cell seeding, and a removable culture insert that can be transferred between modules.

To characterize the development stage and barrier integrity of the produced skin, tetrapolar electrodes were integrated on the chip to quantify the transepithelial electrical resistance in real time. The structure and functionality of the developed model were studied using histological and immunofluorescence staining.

The developed device was successfully used to grow and sustain a physiologically relevant human skin model. Extracellular matrix components were secreted by the fibroblasts and accumulated in the scaffold, recreating the microenvironment of the native human dermis. The use of a scaffold provided the necessary mechanical stability. The co-culture of primary human keratinocytes resulted in a fully differentiated skin equivalent. In the future, this innovative low-cost platform could reduce the dependence on animal models and provide a new *in vitro* system to study skin diseases and evaluate the safety and efficacy of novel drugs and technologies.

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## *In silico* and *in vitro* approaches supporting target organ safety assessment in pharmaceutical drug discovery

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Understanding the factors driving the attrition rate and reducing it remain a key challenge in pharmaceutical drug development. Safety issues still represent about 80% of project closures in pre-clinical phase, and around 40% of drug failures in clinical Phase I. Cardiac, hepatic and neurological toxicities are the leading reasons. Tackling this challenge starts with an early comprehensive evaluation of the potential safety issues associated to the primary target (Target Toxicity Evaluation), followed by an evaluation of undesirable secondary targets (off-target profiling). While most pharmaceutical companies have developed robust safety strategies to de-risk genotoxicity and cardiotoxicity issues, partly driven by regulatory requirements, there is a paucity of well-established and standardized models for derisking safety issues affecting other organs or systems, such as the central nervous system or the liver. To address these risks, a combination of *in silico* and *in vitro* assays has been put in place from the earliest stages of drug discovery, to help chemists designing safer molecules, and to select the best compounds to progress into animal studies, thereby reducing the total number of animals used in a project. Whenever possible, the use of human-derived tissues or cell lines is favored. In addition to this de-risking strategy routinely applied to all our new chemical entities, mechanistic investigations can be deployed to solve project-specific safety issues, using fit-for-purpose assays. Ultimately, understanding the predictive value of the *in silico* and *in vitro* tools for non-clinical safety testing and their translatability to humans will enable to optimize assays in order to address the key objectives of a safety strategy, i.e., hazard identification, risk assessment, and mitigation.

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## Read-across can increase confidence in the next generation risk assessment for skin sensitization: A case study with resorcinol

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Historically skin sensitization risk assessment for cosmetic ingredients was based on animal models, however regulatory demands have led to Next Generation Risk Assessment (NGRA), using data from New Approach Methodologies (NAM) and Defined Approaches (DA). This case study was meant to investigate if the use of resorcinol at 0.2% in a face cream was safe and a maximum use concentration could be defined. As a first step existing information for resorcinol was collected, comprising physicochemical properties (molecular weight, volatility, logP, pH), *in silico* (TOXTREE, TIMES-M, TIMES-P), *in chemico* (DPRA) and *in vitro* (KeratinoSens™, U-SENS™, SENS-IS) data. The tier 1 hazard prediction by the sequential testing strategy DA identified resorcinol as skin sensitizer (Cat. 1) with a high probability of 92%. The subsequent tier 2 potency prediction yielded equivocal Cat. 1B that could not provide sufficient confidence to determine a point of departure (POD). Therefore, read-across was applied to increase the level of confidence. Analogue searches in various tools and databases using “mode of action” and “chemical structural features” retrieved 535 analogues. After excluding analogues without a defined structure, similar reactivity profile and skin sensitization data, 39 analogues remained. The final selection was based on three approaches: expert judgment, chemical similarity or Local Lymph Node Assay data (LLNA). As already identified by the DA, all read-across approaches predicted resorcinol as a skin sensitizer and in addition indicated a moderate potency. A POD derived from the LLNA EC3 of 3.6% was determined leading to a favorable NGRA conclusion and a maximum use concentration of 0.36%. This was confirmed by a traditional risk assessment based on the available animal data for resorcinol.

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## Beyond animal testing for skin sensitization of cosmetic ingredients: A case study with propyl paraben

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Skin sensitization is a key adverse health effect to be addressed in the safety assessment of cosmetic ingredients. Since 2013, animal testing has been prohibited in the EU, promoting new approach methodologies without animal testing to be used for a so-called Next Generation Risk Assessment (NGRA). For a skin sensitization NGRA, different non-animal methods have been integrated into our tiered sequential stacking testing strategy Defined Approach (DA). This case study shall illustrate how this DA, by combining several *in silico*, *in chemico* and *in vitro* methods, is able to predict sensitization hazard and potency, thus determining whether the consumer can be exposed to 0.2% propyl paraben (PP) in a face cream. Seven data inputs were integrated, including TIMES-M/P, TOXTREE, pH, volatility, qualitative DPRA, KeratinoSens™ and U-SENS™ using a combination of statistical models. PP was predicted as non-sensitizer with high probability (probability to be a skin sensitizer of 16% well below the cut-off of  $\leq 30\%$ ). Given the positive test results of KeratinoSens™ and U-SENS™, a benchmark approach using structurally related parabens was applied to increase the confidence in the hazard prediction. The DA clearly predicted these parabens as non-sensitizer. Based on animal and human data for the related parabens indicating none or only rare cases of skin sensitization, PP was considered as non-sensitizer supporting the DA outcome.

Thus, the use concentration of 0.2% PP in a face cream was regarded as safe for consumers in terms of the risk of skin sensitization.

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## Bisphenol A, bisphenol F and bisphenol S: The bad and the ugly. Where is the good?

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**Background and Objectives:** Bisphenol A (BPA), a reprotoxic and an endocrine-disrupting chemical, has been substituted by alternative bisphenols such as bisphenol F (BPF) and bisphenol S



(BPS) in the plastic industry. Despite their detection in placenta and amniotic fluids, bisphenols effects on human placental cells have not been characterized. Our objective was to *in vitro* explore and compare the toxicity of BPA to its substitutes BPF and BPS to highlight their potential risks for placenta and then pregnancy.

**Materials and Methods:** Human placenta cells (JEG-Tox cells) were incubated with BPA, BPF and BPS for 72 hours. Cell viability, cell death and degenerative P2X7 receptor and caspases activation, and chromatin condensation were assessed using microplate cytometry and fluorescence microscopy.

**Results:** Incubation with BPA, BPF or BPS was associated with P2X7 receptor activation and chromatin condensation. BPA and BPF induced more caspase-1, caspase-9 and caspase-3 activation than BPS. Only BPF enhanced caspase-8 activity.

**Discussion and Conclusion:** BPA, BPF and BPS are all toxic to human placental cells with P2X7 receptor being a common key element. BPA substitution by BPF and BPS doesn't appear as a safe alternative for human health, more particularly for pregnant women and their fetus.

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## Chemically selective and label-free characterization of pancreas organoids inside hydrogel matrices

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Within the growing field of alternative approaches to animal testing, the implementation of *in vitro* cell cultures in 2D and 3D environments has experienced a surge in popularity over the last decades. The cultivation of three-dimensional cell culture systems, especially organoids, constitutes a promising approach in different fields of life sciences and is gaining interest with regard to clinical applications and personalized medicine. Although the cultivation of complex cellular systems is evolving steadily, their analysis is stagnating, relying on the visual inspection of organoid size and development via light microscopy. While analyzing the expression of specific marker molecules via immunofluorescence staining is a reliable approach to determine the identity of organoids, the fixation and staining procedures of 3D cellular structures in their extracellular matrix (ECM) produces several issues and artefacts. Further, the interaction of the cells with the ECM during organoid expansion and development is completely neglected in established analysis procedures. In order to

develop a comprehensive understanding of organoids non-invasively during their development, confocal Raman microscopy was used in this study to characterize pancreas organoids in different ECMs. The label-free and chemically selective technique was implemented to monitor the interaction of cells with two synthetic hydrogels in order to assess their suitability to replace commercial Matrigel<sup>®</sup>. Using confocal Raman imaging, the contact-free analysis of pancreas cells within the ECM could be achieved, successfully showing the distribution of nucleic acids, protein- and lipid-rich regions within the cell. Further, the expansion mechanism and interaction of whole organoids with the ECM was investigated, monitoring the chemical composition of the surrounding environment as well as the lumen of the organoid in detailed imaging and higher throughput approaches. These results provide a glimpse into the possibilities of confocal Raman microscopy as an innovative technology to revolutionize the analysis of 3D cell culture systems.

**Presentation:** Poster

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## Human *in vitro* models to study bone metastases

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The skeleton is a preferential site for metastatic invasion by cancer cells, especially from prostate and breast cancer. Complications to the bone reduce both length and quality of life of patients in an aggressive way. No cure has been found yet, and the metastatic process is determined by complex interactions between cancer cells and the bone microenvironment. Since osteoblasts (bone forming cells) at the endosteal surface in the bone marrow are the first bone cells to be reached by invasive cancer cells, we aim to unravel the cellular "crosstalk" of metastatic cancer cells and osteoblasts and find new therapeutic routes.

We developed a human co-culture model of differentiating osteoblasts (SV-HFO) and metastatic prostate cancer cells, either bone (PC-3) or non-bone derived (LNCaP). To be distinguished from osteoblasts, cancer cells were lentivirally transduced with GFP (green fluorescent protein). Direct interaction with metastatic cancer cells was studied during 3 weeks of osteoblast maturation. FACS and fluorescence microscopy were used to monitor growth of cancer cells and biochemical assays to study osteoblast differentiation and mineralization. Illumina micro-array analyses were performed after FACS-sort separation of osteoblasts and cancer cells.

Results showed that the stage of osteoblast differentiation is important in cancer cell fate. Both bone and non-bone metastatic



prostate cancer cells did not grow on late stage differentiated osteoblasts, only on early-stage osteoblasts. Bone metastatic cancer cells were able to keep the osteoblasts in this early stage, thereby affecting the bone maturation and stimulating cancer cell growth. Differential gene expression found during these stages revealed new targets for therapeutic intervention.

Testing agents that restore osteoblast maturation and thereby inhibit cancer cell growth in the bone is the promising therapeutic route that we are currently investigating in an adapted format of the current model towards high-throughput screening of FDA approved therapeutics.

**Presentation:** Poster

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## Global effort to end animal testing for health claims of foods and beverages

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Due to growing ethical, scientific, financial, and public relations concerns, the global food and beverage industry is increasingly moving away from animal testing for establishing human health claims to market products and ingredients such as alcohol, cereal, chocolate, juice, pasta, soda, tea, and much more. Thousands of dogs, rabbits, monkeys, pigs, hamsters, mice, rats, and even chimpanzees have been used in these experiments, during which animals have been restrained, stressed, poisoned, lacerated, infected, otherwise harmed and killed. PETA has so far successfully worked with more than 117 companies in this sector worldwide – both those that have already and those that have not yet pursued animal testing for this purpose – to adopt new formal policies against conducting, funding, and commissioning animal tests unless explicitly required by law. PETA is also working with international government entities to remove any explicit requirements, suggestions, and/or acceptance of animal tests in their health claim regulations. In this study, we compare and contrast varying food and beverage health claim regulations from the United States, European Union, Canada, Japan, South Korea, China, and Taiwan. We discuss the scientific limitations of animal tests for some of the most common health claims such as blood pressure regulation, blood lipid control, anti-fatigue, digestion improvement, immune system boost, and joint protection. We also describe non-animal testing methods that are more relevant to human health, and we share our experience in working with companies and government entities to end animal tests for establishing health claims to market food and beverage products and ingredients.

**Presentation:** Poster

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## Evaluation of a human iPSC-derived BBB model for repeated dose toxicity testing with cyclosporine A as model compound

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The blood-brain barrier (BBB) is a highly restrictive barrier that preserves central nervous system homeostasis and ensures optimal brain functioning. Using BBB cell assays makes it possible to investigate whether a compound is likely to compromise BBBs functionality, thereby probably resulting in neurotoxicity. Recently, several protocols to obtain human brain-like endothelial cells (BLECs) from induced pluripotent stem cells (iPSCs) have been reported. Within the framework of the European MSCA-ITN in3 project, we explored the possibility to use an iPSC-derived BBB model to assess the effects of repeated dose treatment with chemicals, using Cyclosporine A (CsA) as a model compound. The BLECs were found to exhibit important BBB characteristics up to 15 days after the end of the differentiation and could be used to assess the effects of repeated dose treatment. Although BLECs were still undergoing transcriptional changes over time, a targeted transcriptome analysis (TempO-Seq) indicated a time and concentration dependent activation of ATF4, XBP1, Nrf2 and p53 stress response pathways under CsA treatment. Taken together, these results demonstrate that this iPSC-derived BBB model and iPSC-derived models in general hold great potential to study the effects of repeated dose exposure with chemicals, allowing personalized and patient-specific studies in the future.

**Presentation:** Poster



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## International harmonization of non-animal methods for biomedical training

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Due to heightened public concerns about the use of animals in experiments, technological advances in simulation, increased institutional financial constraints, and educators' need for better teaching and assessment tools, biomedical institutions around the world are increasingly moving away from animal-based training methods. Millions of dogs, cats, rabbits, pigs, goats, mice, rats, guinea pigs, frogs, and other animals are used in biomedical training every year, during which they are cut into, burned, drugged, otherwise harmed and killed. PETA and its international affiliates have successfully worked with medical institutions around the world – via expert dialogue, meetings with regulators, public campaigns, and groundbreaking industry partnerships – to switch from using animals to instead using more modern humane teaching methods such as computer assisted learning, human patient simulators, virtual reality, and hyper-realistic synthetic tissue models, among others. As a result of these and other sustained efforts, numerous institutions around the world have adopted non-animal methods across a variety of disciplines: K-12 biology and anatomy classrooms, university life science and zoology courses, undergraduate medical training, military and civilian trauma training, chemical casualty drills, pediatric intubation courses, graduate pulmonary programs, obstetrics and gynecology residencies, and more. In this study, we examine the scientific, ethical, and legal considerations for harmonizing non-animal medical training across different facilities globally, describe technological advancements that are more cost-efficient and relevant to human medicine and health, and share our experience in working with various stakeholders to end the use of animals for biomedical training.

**Presentation:** Poster

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## Roadmap to replacement – Addressing surplus animal breeding

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The most recent European statistics on animals used for scientific purposes revealed an apparent downward trend. In 2017, 9.38 million uses were recorded – an 18% decrease from the 2011 total

of 11.5 million. However, closer scrutiny of the data reveals that an additional 12,597,816 animals were bred and killed, not used for any scientific purpose, bringing the actual total animal use for 2017 to almost 22 million. Breakdown of this figure shows an almost equal split between genetically altered (6,113,281 uses) and conventional animals (6,484,535 uses).

It is a concern to note that data collected over the last eleven years in the UK suggest this is a sustained trend – surplus animals comprise half the total animal use, and this has been the case for at least the last seven years. This equates to over 13 million animals bred and killed in the UK alone, despite the introduction of a breeding framework and the ultimate aim of Directive 2010/63/EU to replace animals in scientific procedures. The most recent European statistical report indicates an increase in the use of animals for the creation of new genetically altered strains, and over five and a half million surplus animals bred and killed for the maintenance of genetically altered animal lines.

Here we focus on the extensive overbreeding of genetically altered animals; we analyze research areas where genetically altered animals are used most prominently, document the translational failure of these as models, and offer suggestions to replace animals to develop a more effective, human-predictive research strategy. We outline what is needed to streamline breeding processes and suggest this could be rolled out to animal breeding programs more widely, in order to reduce overbreeding and wasteful culling of animals, until the biomedical research paradigm completes the shift away from animal use altogether.

**Presentation:** Poster

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## Microfluidic platform for development of bio-artificial retina

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Retinal diseases account for major discomfort and loss of vision, highlighting the need for alternative therapeutic strategies and newer drugs. Drug discovery and development for newer molecules alleviating retinal damage requires high throughput screening methods and reliable models for recapitulating the *in vivo* retinal responses. Several animal models, *in vivo* and *ex vivo* explants are being employed for evaluating the drug molecules in pipeline. However, most of these systems are unreliable as they fail to mimic the major physiological factors, like tissue extracellular-matrix and micro-architecture. Thus, the retina-on-a-chip ideology was pursued to address these lacunae and provide an alternative-to-animal, *in vitro*, perfusion-based system



for cultivating retinal cells. A computationally validated microfluidic device was designed and fabricated using PDMS, to support the co-culture of retinal cells from rats, under a continuous flow of medium. This perfusion-based, organotypic co-culture was screened for media optimization and proliferation in order to sustain the cellular shear exerted by the fluid flow. Further, the transepithelial-electrical resistance (TEER) and permeability of FITC-dextran was measured to study the barrier integrity of epithelial cells. The co-culture was screened for retinal markers, as well as the retinal precursor cells were assessed for their photoreceptor characteristic and thereby analyzed for the effect of light exposure. Results indicated that the device demonstrated a high potential as an *in vitro* cellular model for an in depth understanding of the retinal micro-physiology. The model was successfully used to understand the penetration and metabolism of therapeutic molecules, like chemical drugs and biomolecules.

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**Presentation:** Poster

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## Towards the development of animal product-free *in vitro* systems for NGRA of consumer goods

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There is an increasing acceptance of the role *in vitro* assays can play in assuring consumer safety, particularly as part of Next Generation Risk Assessment (NGRA) (Baltazar et al., 2020). NGRA is an exposure-led, hypothesis-driven approach integrating new approach methodologies (NAMs) to ensure chemical safety without the use of animal testing. There is also a growing desire to remove animal products from these *in vitro* assays

to make them more scientifically robust and human-relevant. For example, the use of foetal bovine serum (FBS) and animal-derived antibodies can introduce a lot of batch-to-batch variability potentially resulting in experimental quality (e.g., contamination of FBS; specificity of antibodies) and reproducibility issues (Baker et al., 2016; van de Valk et al., 2018). Additionally, it is more frequently becoming recognised that knowledge of all the constituents of the cell culture medium used and their influence on cellular processes are important for improved reproducibility (Hirsch and Schildknecht, 2019). Therefore, ideally chemically defined media would be used to culture human cells for *in vitro* assays to eliminate any remaining scientific quality issues resulting from use of animal- or human-derived components (van der Valk et al., 2010) although this is technically very challenging. Here we will describe some of the challenges, opportunities and potential options for replacing animal-derived products in *in vitro* systems.

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**Presentation:** Poster

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## World pharmacopeias are ready to adopt non-animal *in-vitro* replacement tests for detection of pyrogens. Are you?

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As the world is increasingly concerned with the ethics of using animals for the detection of Pyrogens in pharmaceutical preparations, manufacturers seek to eliminate animals in these tests. However, current regulations require the testing of all injectable



products for the presence of pyrogens, whose effects range from fever, shock, and in some instances, death.

Current compendial methods are the Rabbit Pyrogen Test (RPT), the Bacterial Endotoxins Test (BET), and the Monocyte Activation Test (MAT).

The RPT used over 400,000 animals in 2015 and this number is still growing today (Hartung, 2015). Ordinarily, rabbits may be reused in the RPT after a brief period. However, modern biological products, such as monoclonal antibodies, may induce an immune response preventing reuse of the rabbit. The *in vitro* replacement for the RPT is the MAT, as it detects any substance that would be pyrogenic in humans. The assay uses human monocytes that release inflammatory cytokines upon pyrogenic stimulation, proportionally to the total amount of all pyrogens in a sample. The released cytokines are quantitated using an ELISA assay system.

The BET or LAL test has been in effect since the early 1980s. The test uses the amoebocytes from the blood of *Limulus polyphemus* and *Tachypleus tridentatus*. The populations of *Limulus* are stable in the USA due to a highly regulated fishery while the populations of the three Asian species have been decimated. As a result, *T. tridentatus* is now listed as an endangered species by the IUCN (Laurie et al., 2019).

The replacement for the LAL test is the rFC assay initially developed to eliminate the need to draw blood from living animals. This test was firstly adopted by the European Pharmacopoeia to become effective in January 2021.

This presentation discusses *in vitro* replacements for pyrogen testing, actions taken to incorporate those replacements and remaining constraints for implementation.

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**Presentation:** Poster

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## Confirmatory preclinical studies as means to guide decisions to engage in clinical trials: The decide project

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In preclinical research, we typically explore potential novel therapies with the ultimate goal of clinical translation. In a first step, exploratory studies aim to uncover possible, yet unknown pathophysiological relationships. During confirmation, researchers generate incremental experimental evidence that reduces uncertainty about investigated effect(s). Thus, confirmatory research potentially corroborates an initial hypothesis by replicating results, strengthening the validity and identifying boundary conditions. This will mitigate the risk of failure for translation. To develop new therapies in a responsible way, an increased and critical scrutiny of findings from preclinical studies is mandatory and resources (e.g., scientific personnel, funding as well as patients' samples, laboratory animals or materials) need to be used efficiently.

To investigate the processes and specificities of confirmatory research, the DECIDE project (Decision-Enabling Confirmation of Innovative Discoveries and Exploratory Evidence) will monitor, counsel, and evaluate the scientific process and outcomes of preclinical confirmatory research projects over four years. The preclinical projects come from diverse biomedical disciplines and include studies across multiple sites. Within the scope of the project, we will

- (i) support preclinical confirmatory studies through continuing education towards high methodological rigor,
- (ii) develop a flexible framework for planning, conducting, analysing, and evaluating confirmatory research,
- (iii) refine this framework through communication with the research community, leveraging a meta-analysis with the results of these projects; and
- (iv) prepare means to facilitate dissemination and sustainability of the framework.

The overall goal of the DECIDE project is to develop recommendations on best practice for confirmatory studies to help increasing the evidence, robustness, and prediction of preclinical research.

**Presentation:** Poster



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## Evaluating dog use in biomedical research in order to identify non-animal, human-based replacement options

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For the past decade, over 60,000 dogs have been used annually for research and testing in the US. Publicly accessible data are not available to evaluate the numbers of dogs used specifically for biomedical research in the US, but trends elsewhere suggest up to 68% may be used for basic, translational and applied research purposes – at least 40,000 dogs per year.

We searched the National Institutes of Health REPORTER database for projects using dogs between 2015 and 2019, in order to gain a clearer picture of how dogs are currently used in biomedical research funded by the U.S. Public Health Service. We evaluated the purposes of the research, total costs, the number of dogs used, procedures they underwent (including euthanasia), and tried to assess the potential benefits to human health as well feasibility of non-animal approaches.

Of 679 projects that mentioned dogs in the abstract, 388 were actively using dogs, 188 of which were classified as biomedical research with total funding of over 83 million USD. Cancer research was the most common use of dogs – however deeper analysis indicated that over 60% of these projects were employing client-owned companion dogs (parallel patient population). In contrast, 37 projects for cardiovascular disease research, an area which almost exclusively used purpose-bred dogs, often involving pain and distress, were awarded over 57 million USD. Questions are being raised about the validity of some canine models of research, including cardiac studies, and relevant non-animal replacement methods are rapidly evolving.

Overall, our research provides details of federally funded research using dogs, allowing investigation into the validity of canine models that seek to address human health concerns and indicating where non-animal methods are superior and could replace dogs altogether.

We propose a roadmap enabling replacement of dogs as models of human disease with human-based approaches.

**Presentation:** Poster

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## Retrospective evaluation of the acute fish toxicity test for pesticide registration

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The acute fish toxicity test is used to assess the potential risk of substances to aquatic organisms. In the United States (U.S.), the test is typically conducted in three different fish species: a cold and a warm freshwater species and a marine/estuarine species. Therefore, three separate acute fish toxicity tests are conducted for a single chemical, with each test potentially requiring 200 or more fish per chemical. We conducted a retrospective analysis of data submitted to the U.S. Environmental Protection Agency (EPA) to support pesticide registrations to determine whether reducing the number of species tested would impact the ability of pesticide risk assessments to identify and characterize potential risks to fish. Lethal concentration 50% (LC50) values and experimental details were extracted and curated from 762 acute fish toxicity studies submitted to EPA for pesticide registrations. From this data set, 87 substances had data from studies in three species that were considered acceptable based on defined criteria. Data were analyzed to determine any trends among species in terms of relative differences in acute toxicity LC50 values. Where a difference could be detected, coldwater species were most often the most sensitive species (26/45); for the remaining 42 substances there was no clear difference in species sensitivities. These results suggest that it may be possible to use data from as few as one species, thereby substantially reducing the number of fish required while still meeting risk protection goals.

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**Presentation:** Poster



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## A novel system of infusion to provide drugs within *ex vivo* skin models

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The development of new models predicting human responses to biotherapeutics is a major challenge in the field of bioengineering. In particular, how high molecular weight molecules diffuse in human tissues is still elusive.

In this study, we integrate and characterize a novel microfluidic system of infusion to provide drugs within *ex vivo* skin models. The resulting FlowSkin model is based on the implantation of a porous catheter into a human skin biopsy embedded in a matrix and maintained in physiological conditions.

FlowSkin models have been cultivated and infused in standard culture conditions for 10 days. During this period, exchanges between the flow and the tissue were maintained without detectable alteration of tissue viability, while preserving physiological levels of cell proliferation. The diffusion of molecules exhibiting different molecular weights has been investigated by a combination of X-ray tomography and Light-Sheet Fluorescence Microscopy. Biological effects on the infused skin models upon administration of a bioactive compound through the flow have been carefully investigated. The obtained results position the FlowSkin model as a new relevant tool to study the efficacy and/or toxicity of intravenously administered drugs directly on human skin.

**Presentation:** Poster

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## New methodology implementation for *in vitro* micronucleus analysis in a CRO (Bioagri Laboratório Ltda – Merieux NutriSciences): Comparison of conventional microscopy and flow cytometry

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*In vitro* micronucleus (MN) analysis is extremely important within the regulatory agency scenario, based on Guideline OECD 487 (2016), the results obtained following its guidelines and good laboratory practice (GLP) procedures determine whether the test

items are genotoxic or not. This analysis should be performed in several methods, including conventional microscopy (Rodrigues et al., 2018). On the other hand, the automated methods result in increased sensitivity and combined with their rapidity. Such details are interesting when it is said that it is related to production in a Contract Research Organization (CRO). In this sense, our laboratory verified the positive response criterion in relation to vehicle control (VC) between the two methodologies (microscopy vs flow cytometry) in order to ensure the results safety. For this, 30 assays were performed under three conditions (short exposure with and without metabolic activation and long exposure without metabolic activation) using the CHO-K1 cell line based on OECD 487 (2016). The MN analysis was using microscopy and flow cytometry, in which it was used Microflow *in vitro* (Litron) commercial kit. Our results showed a 6-fold increase in the MN amount in relation to VC when microscopy was used. As expected, flow cytometry demonstrated an increase in the detection MN capacity, on average 11-fold, depends on condition and positive control concentration, since it is possible to discriminate it from nucleus according to the fluorescence intensity (Bryce et al., 2013). In addition to the high sensitivity, this methodology requires less material than conventional reading, being an excellent technology and alternative, in front of our need for fast and reproducible results.

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**Presentation:** Poster

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## Application of human induced pluripotent stem cell cardiomyocytes (hiPSC-CM) in preclinical *in vitro* cardiotoxicity assessment: Challenges and opportunities

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Drug-induced cardiotoxicity is an important safety concern in clinical trials and a cause of drug withdrawal. Therefore, it is critical to identify cardiotoxic compounds early in drug development. hiPSC-CMs and the xCELLigence® Real Time Cell Analyzer (RTCA) Cardio Extra-Cellular Recording (ECR) technology enable collection of contractility, electrophysiology and cell



viability data to perform an integrated assessment of changes in cardiomyocytes function upon treatment with compounds. Our objective was to test proprietary compounds in hiPSC-CM and compare with the data generated *in silico*, *in vitro* and *in vivo*.

For several compounds exhibiting strong *in vitro* hERG inhibition, *in silico* action potential duration (APD) prolongation and the QTc prolongation in anesthetized guinea pigs, we observed field potential duration (FPD) prolongation and EAD (early after depolarization) events in hiPSC-CM. Good correlation was observed between beat rate and contractility amplitude in hiPSC-CM and heart rate and contractility in anesthetized guinea pigs. For a few compounds electrophysiological changes observed in hiPSC-CM did not directly translate to *in vivo* effects, which would require a more thorough analysis to understand the discrepancies.

Overall, using hiPSC-CM with RTCA Cardio ECR system have several advantages for preclinical safety assessment: relatively easy to manipulate and quick turnaround time which allows pre-selection/characterization of the compounds before *in vivo* studies; a simultaneous collection of many parameters regarding cardiomyocytes function; potentially a better translation to the clinic than animal cells. On the other hand, there are some challenges that should be considered when using this cell model: hiPSC-CM have immature phenotype compared to adult cardiomyocytes; using a monocellular culture might not be sufficient to capture all cardiotoxic effects; high variability of the results between the experiments, different cell models or laboratories makes it difficult to establish a widely accepted cut-off criteria.

**Presentation:** Poster

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## Defining the reproducibility and applicability domain of devTOX quickPredict, a human pluripotent stem cell-based developmental toxicity assay

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To establish confidence in new approach methods (NAMs) and enable their use in a regulatory setting, it is necessary to assess a NAMs accuracy and reproducibility, as well as define its applicability domain, in terms of both chemical and biological space. Over 100 chemicals have been evaluated with the devTOX quickPredict (devTOXqP) assay, which predicts the developmental toxicity potential of a chemical based on changes in human iPS cell metabolism. The assay predicted the developmental toxicity potential across this diverse set of chemicals with 87% accuracy (88% sensitivity, 86% specificity). Within individual chemical classes (i.e., pharmaceuticals or pesticides), assay accuracy ranged from 81% to 94%, demonstrating the broad applicability of the assay. To understand the applicability domain of the assay, the results were separated into different pharmacological categories and performance was assessed. The assay's sensitivity in the different pharmacological categories ranged from 50% to 100% and provides insight into the assay's biological applicability domain. For example, developmental toxicants classified as kinase modulators were predicted as correctly with 100% sensitivity, whereas receptor modulators were predicted with 50% sensitivity, which was reflective of receptor expression in iPS cells. The reproducibility of the predictive model was evaluated using independent replicates of three chemical treatments (carbamazepine, methotrexate, thalidomide) conducted by multiple technicians with two iPS cell lines over the course of 5 years. The interpolated developmental toxicity potential (dTP) values (determined using the devTOXqP predictive model) were within two standard deviations of the mean for each of the chemicals, demonstrating that the assay endpoints are reproducible over time. These data demonstrate the importance of understanding a NAM's biological system, its strengths and its limitations. Taken together, these data demonstrate the accuracy, reproducibility and broad applicability domain of the devTOXqP assay and support its use as an alternative to animal models for developmental toxicity testing.

**Presentation:** Poster



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## Ethical and scientific concerns regarding the continued use of experimentally induced brain injuries in primates

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For more than a century, researchers have been deliberately inducing permanent, debilitating, sometimes fatal brain injuries in non-human primates for the purported purpose of gaining insight into the neural bases of human behavior. While these experiments have caused extreme suffering and death for countless primates kept in laboratories, their contribution to our accurate understanding of human brain-behavior relationships has been limited, at best. Confounds introduced by the negative physiological and psychological impact of laboratory life on sensitive and intelligent animals, critical species differences in neurodevelopment, neuroanatomy and neurochemistry, and oversimplified behavioral assays are just a few of the problems impacting the validity of data from these experiments. Furthermore, considerable advances in our ability to study human brain structure and function *in vivo*, including multivariate algorithms for lesion-symptom mapping in humans, transcranial magnetic stimulation, and non-invasive neuroimaging and electrophysiological recording techniques have rendered these harmful procedures obsolete and needless.

Despite the inherent cruelty, limited utility, and lack of necessity, numerous laboratories still induce brain lesions in our fellow primates. We will present data on the current prevalence, purpose, and cost of these procedures in federally funded research in the United States and describe superior alternative research methods available to replace these ongoing experiments. We will also discuss the numerous ethical and scientific arguments for the discontinuation of these methods in contemporary neuroscience research.

**Presentation:** Poster

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## A targeted metabolomics-based assay using human induced pluripotent stem cell-derived cardiomyocytes identifies structural and functional cardiotoxicity potential

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Implementing screening assays that identify functional and structural cardiotoxicity earlier in the drug development pipeline has the potential to improve safety and the cost and time required to bring new drugs to market. In this study, a metabolic biomarker-based assay was developed that predicts the cardiotoxicity potential of a drug based on changes in the metabolism and viability of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM). Assay development and testing was conducted in two phases: (1) biomarker identification and (2) targeted assay development. In the first phase, metabolomic data from hiPSC-CM spent media following exposure to 66 drugs was used to identify biomarkers that identified both functional and structural cardiotoxicants. Four metabolites that represent different metabolic pathways (arachidonic acid, lactic acid, 2'-deoxycytidine, and thymidine) were identified as indicators of cardiotoxicity. In phase two, a targeted, exposure-based biomarker assay was developed that measured these metabolites and hiPSC-CM viability across an eight-point concentration curve. Metabolite-specific predictive thresholds for identifying the cardiotoxicity potential of a drug were established and optimized for balanced accuracy or sensitivity. When predictive thresholds were optimized for balanced accuracy, the assay predicted the cardiotoxicity potential of 81 drugs with 86% balanced accuracy, 83% sensitivity, and 90% specificity. Alternatively, optimizing the thresholds for sensitivity yields a balanced accuracy of 85%, 90% sensitivity, and 79% specificity. This new hiPSC-CM-based assay provides a paradigm that can identify structural and functional cardiotoxic drugs that could be used in conjunction with other endpoints to provide a more comprehensive evaluation of a drug's cardiotoxicity potential.

**Presentation:** Poster



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## 3Rs Centre in laboratory animal science in Sri Lanka

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3Rs Centre was founded in 2019 at the Faculty of Medicine, University of Colombo to promote and facilitate alternative models to replace animals use (Replacement) in education (skills development) and research. This reduces the number of animals (Reduction) bred and use for research purposes leading to reduction of animal suffering (Refinement). Necessary measures were already taken to introduce several alternative models for research and skills development.

**Presentation:** Poster

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## Integrated strategy for eye irritation assessment of agrochemical formulations

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For the registration of agrochemical formulations, acute eye toxicity assessment is required by regulatory agencies. The Draize rabbit eye test (OECD TG 405) has worldwide acceptance to assess eye irritation, even it has been increasingly questioned. The test distinguishes four categories considering reversible and non-reversible ocular lesions according to UN GHS Category 1 (severe eye damage), 2A and 2B (reversible eye damage) and No Category (minimal effects).

Bovine Corneal Opacity and Permeability (BCOP) and Short Time Exposure (STE) are methods for identifying Cat. 1 and No Cat. products, according to OECD TG 437 and TG 491 respectively. The limitation of both methods is to classify in the middle-range categories (2A and 2B). The aim of this work was to integrate the results of different methodologies to identify these categories.

The BCOP and STE methods were used to test 17 pesticides manufactured by ATANOR SCA. These products had been previously classified in categories 1, 2A, 2B or No Cat. using the Draize eye irritation test.

Using the BCOP test, liquid products were tested neat and at 10% and then corneal histopathological analysis was performed.

By STE test, all formulations were tested at 5% and 0.05%. It is known that BCOP and STE methods have low false negatives to classify No-Cat. products. The STE also has low false positives to classify Cat I. Taking into account the sensitivity and specificity of each method, we have distinguished the products into three categories: 1, 2A+2B and No Cat. with an accuracy of 76.5% (13 of 17). The remaining 23,5% corresponds to products whose damage was overestimated with respect to the *in vivo* test (there were no cases of low estimation).

Finally, we successfully established an in-house strategy for testing agrochemicals eye irritation.

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**Presentation:** Poster

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## CRISPR-Cas9 gene editing in cultured fish cells – Toward a new era of mechanistic toxicology

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In environmental toxicology, adverse outcome pathways (AOPs) provide mechanistic insights into toxicity pathways, spanning multiple levels of biological organization, from cell to organism to entire populations. On the cellular level, chemicals can exert a toxic response through a variety of molecular mechanisms. However, the genes associated with these molecular mechanisms are not fully characterized, preventing the generation of reliable AOP networks. The CRISPR-Cas9 gene-editing system constitutes a well-suited instrument to interrogate the genes involved in toxicity pathways on a cellular level. In this work, we established a CRISPR-Cas9 gene-editing platform in the rainbow trout (On-



corhynchus mykiss) intestinal cell line (RTgutGC) based on the electroporation of ribonucleoprotein (RNP) complexes consisting of the Cas9 protein and tracr/crRNA duplex targeting *cyp1a1*, a gene encoding for one of the main enzymatic components of the Cyp P450 complex in fish. T7 endonuclease assay, Sanger sequencing and bioinformatic analysis indicated the presence of insertions and deletions of variable length in the *cyp1a1* gene region targeted by the CRISPR/Cas9 machinery. Finally, a method for the derivation of clonal cell lines was established for RTgutGC cells, allowing the isolation of two pure *cyp1a1* clones bearing 1 bp and 101 bp insertions, respectively, disrupting the open reading frame. Overall, our gene-editing system in RTgutGC cells has the potential to revolutionize the field of environmental toxicology by offering molecular insights into toxic reactions in fish cells and an alternative method for chemical toxicity testing in live animals.

**Presentation:** Poster

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### Application of *in silico* tools developed within life-VERMEER: Food contact materials as case study

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Worldwide, the interest to substitute harmful chemicals continues to grow in industry, NGOs and the public sector. However, the substitution of these chemicals poses a number of challenges as the candidate compound for substitution may have its own risk profile. Thus, multiple assessments need to be done to address the different possible human health or environmental hazards, the persistence or bioaccumulation potential of the candidate compound, and this by using various exposure scenarios. At present, a series of tools has to be applied to perform these assessments, resulting in both practical and theoretical difficulties, as the individual parts of the evaluation may not cover the complete picture. Development of appropriate tools and methods to support the substitution process, together with adequate guidance on their use is therefore urgently needed. Within the LIFE VERMEER project, a comprehensive platform has been developed integrating existing software for environmental and human health risk assessment. More specifically, the platform consists of the new user-friendly LIFE SPHERA tool which combines the MERLIN-Expo tool for multi-stressors, multi-target and multi-route exposure with the VEGA tool for multi-endpoint hazard

assessment. In addition, ToxEraser, based on the identification of structural alerts by ToxRead, has been developed and included as a tool to suggest candidate substances for risky chemicals. Both LIFE SPHERA and ToxEraser are currently being applied for different case studies. Here, the results obtained for the Food Contact Materials (FCM) case study are presented. For this case study, a migration model for plastic FCM was developed and linked to the hazard predictions made in VEGA within the LIFE SPHERA tool. In addition, a database containing substances used in all FCM types was compiled and used to identify those FCM substances with the highest priority for substitution and to propose possible candidate compounds.

**Presentation:** Poster

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### Identifying putative modes-of-action for environmental chemicals using high-throughput phenotypic profiling

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The United States Environmental Protection Agency (USEPA) is exploring the use of high-throughput profiling methods for rapid bioactivity screening of environmental chemicals. In phenotypic profiling using the Cell Painting assay, cell organelles are labeled with multiple fluorophores to quantify morphological changes of cells upon chemical exposure. While it is well known that drug-like chemicals can be grouped by mode-of-action (MOA) based on the phenotypic profiles, here we explore whether this is also true for environmental chemicals (i.e., pesticides, industrial chemicals, etc.) that might lack a primary target in human cells.

To this end, we screened 120 chemicals with annotated MOAs ("reference chemicals") and 441 environmental chemicals at 8 concentrations (1/2 log<sub>10</sub> spacing, 4 biological replicates) in U-2 OS cells. Cells were exposed for 24 h, fixed and labeled with fluorescent dyes to visualize multiple organelles: nucleus, nucleoli, endoplasmic reticulum, Golgi, actin cytoskeleton, plasma membrane and mitochondria. 1300 features were extracted per cell to construct profiles, which were then compared to each other using Pearson correlation.

Among the reference chemicals, multiple distinct profiles were observed. For example, various DNA damaging compounds (alkylators, topoisomerase inhibitors, antimetabolites) have similar profiles, while microtubule modulators had a different, characteristic profile. Among all tested chemicals, approximately 16 clusters were observed, and 141/441 environmental chemicals



clustered with reference chemicals, indicating that they produce similar phenotypes and hence might have similar cellular effects. A group of strobilurin fungicides had a common profile related to changes in mitochondrial features, consistent with strobilurin's MOA to inhibit mitochondrial respiration in fungi.

Overall, these preliminary results indicate (1) that our screening strategy yields distinct phenotypic profiles and (2) that environmental chemicals may be grouped using these profiles to identify putative MOAs. This abstract does not reflect USEPA policy.

**Presentation:** Poster

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## Evaluation of skin barrier properties of cosmetic ingredients using reconstructed human epidermal model

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Skin barrier is located at the outermost layer of the epidermis, and is an important shield protecting the body from excessive transepidermal water loss (TEWL) and invasion of external factors such as antigens, infectious agent, etc. In understanding the function of the barrier in normal skin and skin diseases, 3D skin models are impactful tools for *in vitro* studies. The purpose of this study is to use 3D skin model in evaluating the effectiveness of cosmetic ingredient in term of moisturizing effectiveness and skin barrier restorative capabilities.

The reconstructed human epidermal model (RHE) has similar morphologies, characteristics, and even biochemical marker expressions comparable to native human skin. In this study, we evaluated the effects of ceramide, which is known to improve skin barrier function, through its skin barrier restorative capabilities observed with RHE. RHE was damaged by SDS treatment, and then treated with four types of ceramides. The restoration of skin barrier function on RHE was assessed by the measurement of TEWL and immunohistochemical staining of epidermal expression markers.

SDS treatment to RHE increased the TEWL value (152%) and decreased the expressions of proliferation and differentiation markers such as p63, CK14, CD44 and filaggrin. When ceramides were applied on RHE pretreated by SDS, TEWL value effectively decreased, and the expression of proliferation and differentiation markers increased.

These results show that RHE can be used as a useful tool in evaluating skin barrier function and determining moisturizing effectiveness.

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**Presentation:** Poster

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## International approaches to implementing alternative test methods for marine biotoxins in shellfish

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The mouse bioassay (MBA) or biological method is commonly used to test for marine biotoxins in shellfish in many parts of the world. Authorities such as the World Health Organization, European Food Safety Authority, and US Food and Drug Administration have noted its lack of sensitivity, specificity, and precision. For scientific as well as ethical reasons, some countries, such as Canada and New Zealand, no longer use the test for routine toxicity testing of shellfish, and as of 2019, the use of the test is effectively prohibited in the European Union as a result of Directive 2010/63/EU. However, other countries still commonly use the MBA. Here, we consider the availability and advantages of alternative test methods for marine biotoxin testing in shellfish. We analyze the capabilities of different methods to protect public health, their regulatory acceptance in various countries, and what resources are needed for their implementation. We also highlight the reasons that some countries transitioned to non-animal methods of marine biotoxin detection, reasons that others continue to use the MBA, and steps being taken in some countries to increase the use of non-animal methods.

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## A full thickness long term skin equivalent allows repeated testing of cosmetics to evaluate efficacy and safety

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**Introduction:** Most commercial 3D *in vitro* epithelial model systems are currently limited by their relatively short longevity that ranges from 3-5 days meaning that long-term or repeat-dose chemical testing over prolonged periods is not possible. There is currently no safe cost-effective way to assess the allergenicity of novel compounds. The Patch Test creates patient discomfort and can trigger anaphylactic shock.

**Objectives:** This study aims to develop a safe and robust predictive method for allergenicity testing of cosmetic compounds and cosmetics.

**Materials and Methods:** Normal human dermal fibroblasts (HDF) were isolated from biopsies and cultured to generate their own native connective tissue scaffolds. After the 28-day development of fibroblast-derived matrices (FDM), hTERT-1 skin keratinocytes were seeded on to the surface.

**Results:** Tissue engineered Fibroblast derived matrices (FDM) produced a self-assembled organized, collagen fiber meshwork with a thickness over 100 µm. This results in models histologically resembling human skin tissue and displayed classical expression of AE/13, collagen type iv and E-cadherin. These equivalents remained viable for at least 42 days in culture as observed by metabolism assay. Immune cells (MUTZ-3 or THP-1) have been added in order to predict allergic potential of cosmetic ingredients. Several contact sensitizers and UVB effect have been tested upon repeated exposure. We have also tested whether antioxidant can reverse the detrimental effect of repeated exposure to sensitizers and UVB by measuring cell viability and transepidermal electric resistance (TEER). Intercellular communication was also observed by live cell imaging in a non-invasive manner.

**Conclusion:** This novel skin equivalent provides physiologically relevant, flexible yet robust *in vitro* tools for use in pharmacological and cosmetic chemical toxicity testing, modelling disease processes and drug absorption studies. It allows repeated testing and study immune response in a non-invasive manner.

**Presentation:** Poster

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## The changing face of chemicals legislation in India: Opportunities to minimize testing on animals

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A new legislative framework for chemicals in India has long been anticipated and, last year, the Indian Ministry of Chemicals and Fertilizers released the draft Chemicals (Management & Safety) Rules, 20XX. The draft Rules include provisions for the creation of a national chemical inventory; the registration, prioritization, and restriction of chemicals imported into or manufactured in India; the implementation of a hazard-communication system; and a safety/chemical accident prevention program.

Modernization of the chemicals legislation provides India with an opportunity to use and promote the most scientifically advanced non-animal assessment strategies, allowing resources to be saved and the lives of countless animals to be spared.

International chemicals legislation, such as the Registration, Evaluation, Authorisation and Restriction of Chemicals regulation in the European Union (Regulation (EC) No 1907/2006) and the US Toxic Substances Control Act (2019) include requirements to avoid the use of animal tests wherever possible, and India must implement similar requirements to ensure it is aligned with international standards. Therefore, PETA India made recommendations to the Indian government highlighting several opportunities for using non-animal testing approaches that protect



human health and the environment, which have now been adopted in the draft Rules (Chemical Watch, 2020). These include, the mandatory use of valid non-animal methods, testing on vertebrate animals only as a last resort, consideration of existing scientific evidence, acceptance of data submitted in foreign jurisdictions, approval of testing strategy prior to conducting any new tests and promotion of a “risk-based assessment” approach.

PETA India continues to work with the Indian government to incorporate additional opportunities to ensure the new chemicals legislation is the most scientifically advanced in the world. This presentation provides the blueprint that will ensure better protection of human health and the environment while promoting the use of reliable, relevant non-animal methods.

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**Presentation:** Poster

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## Development of an ex-vivo retinal dystrophy model by light induced neurodegeneration

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**Purpose:** The aim of this project was to establish a reactive oxygen species (ROS) induced degeneration model on *ex-vivo* porcine retinae. The retina, like all neuronal tissue, is particularly susceptible to oxidative stress because of its high consumption of oxygen, its high proportion of polyunsaturated fatty acids, and its exposure to visible light (Yildirim et al., 2011; Fang et al., 2017). Retinal diseases such as age-related macular degeneration (AMD) or retinitis pigmentosa (RP) lead to neurodegeneration and loss of photoreceptors, which ultimately leads to vision loss and blindness. Although there is strong evidence that photochemical oxida-

tive stress plays a role in AMD and RP pathogenesis as well as in the pathogenesis of several other diseases, there is so far no suitable *ex-vivo* model (Youssef et al., 2011; Hartong et al., 2006).

**Methods:** Primary retinal cells and retinal organ cultures are exposed to blue light in varying intensities and for different periods of time.

ROS production was measured, and the apoptotic state of the retinal cells and organ cultures was determined. Furthermore, the degree of degeneration was determined by means of specific markers using immunohistology, western blot and qRT-PCR. On the one hand, cell-specific markers were used, on the other hand markers for cell death, cellular stress as well as disease-specific markers.

**Results:** We successfully established a disease model with blue-light-induced retinal (neuronal) degeneration which can be used to test novel therapeutic approaches *ex-vivo* in a setting much closer to the human condition, as the morphology of the pig eye is more similar to the human eye than the rodent eye (Hendrickson and Hicks, 2002). Furthermore, organ culture combines the advantages of cell culture experiments: highly reproducible system, which is very standardizable, and a large number of samples can be obtained without major restrictions and *in-vivo* experiments without needing to kill animals for the experiments, as the eyes are a waste product from food production.

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**Presentation:** Poster

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## Investigating the epithelial barrier in human skin 3D tissue models with a non-invasive fluorescein leakage assay

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One of the main functions of the skin is to protect the body from external, potentially harmful influences. For biomedical research, 3D *in vitro* skin models of varying complexity have been generated. However, an intact barrier function is often not verified be-



fore the examination, which can lead to misinterpretation of the results obtained. To investigate this epithelial barrier *in vitro*, we use an innovative technique to create full skin 3D tissue models, namely cell crowns covered with a biological vascularized scaffold (BioVaSc-TERM®). In order to test the integrity of the barrier before using models in downstream applications, we are developing a non-invasive, indirect assay employing fluorescein as a marker molecule. Fluorescein, as a well-characterized validation substance, does not damage the cells when passing paracellularly through human epithelial barriers. In case the barrier is not properly developed, fluorescein leakage is increased, thus providing an indirect measurement of the tightness and, moreover, the functionality of the skin model.

In short, a biological decellularized matrix (SISMuc) is reseeded with primary human fibroblasts and keratinocytes in a co-culture. On day 19 of development, model tightness is estimated by applying solely fluorescein to the skin model. The leakage into the basal compartment is measured time-dependently up to 1h after application. If the model is then judged to be intact, we apply three well-characterized substances on d21, which will later serve as reference substances. These are caffeine as non-irritant but penetrating, SDS as irritant and penetrating and HCl as corrosive substance.

This approach might serve as a further replacement for the Draize skin irritation test (OECD Guideline 404) and a refinement for test methods based on reconstructed human epidermis (OECD Guideline 439) to a more natural full-skin model. Furthermore, we are working on additional non-invasive techniques for model characterization, e.g., 2-photon microscopy or an optimized TEER-device.

**Presentation:** Poster

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## New insight into the mechanisms underlying 5-fluorouracil-induced intestinal toxicity by establishing transcriptomic responses in exposed human intestinal organoids

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5-fluorouracil (5-FU) is a widely used chemotherapeutic agent that has been associated with acute intestinal toxicity, leading to diarrhea, nausea, abdominal pain, and impairment of patients' quality of life. Consequently, cancer therapies are often interrupt-

ed, or dosing has to be lowered, which negatively impacts survival rates. As our understanding of the molecular mechanisms underlying 5-FU intestinal toxicity is limited, prevention of treatment side effects is difficult. Therefore, this study aims to elucidate molecular mechanisms of 5-FU induced GI-toxicity by establishing the associated transcriptomic responses and cytotoxicity using organoids as a novel 3D *in vitro* model of the intestines. *In vitro* human small intestine (SI) and colon organoids were established in a 3D extracellular matrix and exposed to 0, 10, 100, 1000  $\mu$ M of 5-FU. Doses of 5-FU were selected based on physiologically based pharmacokinetic (PBPK) model simulations of clinically relevant dosing regimens for cancer patients. Following exposure, cell viability and apoptosis were assessed as functional endpoints, as well as gene expression profiles by performing RNA sequencing. Based on transcriptomic analysis of differentially expressed genes (DEGs), the most prominent molecular pathways were identified, including cell cycle, DNA damage/repair, p53 signaling, mitochondrial ATP synthesis, metabolism and apoptosis, demonstrating time and dose effects. Short time-series expression miner (STEM) was used to further explore gene alterations, which identified novel, tissue specific mechanisms affected by 5-FU. For colon this includes biosynthesis and transport of small molecules, and mRNA translation mediated by eukaryotic translation termination factor 1, ETF1. In turn, cell signaling mediated by Rho GTPases and FoxO were observed in SI. These results show the potential relevance of human organoid models in the safety assessment of future drugs in development. Insights into possible 5-FU toxicity mechanisms using this human relevant *in vitro* model may lead to the improved prediction of drug-induced damage in human intestines.

**Presentation:** Poster

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## Refining behavioral management programs for research pigs

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Housing and care programs for laboratory pigs have primarily been developed based on cost, convenience of staff, and health status of animals, but with limited consideration of comprehensive animal behavioral management (ABM) programs. Currently, there are many recommendations for improving the well-being of commercial pigs held on farm; however, there are few papers or recommendations specifically about enhancing the behavioral management of research pigs of different breeds. Capitalizing on 2019 as the lunar "Year of the Pig", we formed a Pig Welfare Working Group with the goal of improving research pig behavioral management, with special consideration given to



minipigs. Our primary areas of consideration were enhancing options for pigs to express natural behaviors such as rooting, chewing and foraging; providing comfortable housing with exercise and opportunities for social interaction; increasing use of operant conditioning techniques to better prepare pigs for studies; employing fear-free methods of handling, restraint, transportation, and conduct of technical procedures; and inserting periodic welfare assessments to ensure the program is suited to meet each pigs' needs. While principally designed to implement within our facilities in the EU and North America, these guidelines for a research pig ABM program can be used as a best practice framework that other organizations and facilities can adopt and modify to meet their own program-specific needs.

**Presentation:** Poster

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## Avatars of animals and humans: 3D interactive holographic models

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To date, the education and training of veterinarians, human doctors and researchers is still largely based on the use of live animals. Only in the Netherlands, yearly more than 22,000 animals are used for training and research.

Virtual reality (VR) and Augmented reality (AR) offer unlimited and standardized specimens for dissection. Introduction of this new technology aims to decrease the number of animal and human specimens used for teaching and training.

By substituting animals and humans with dynamic holographic 3D models (Avatar models), we aim to study and acquire anatomic, physiologic, pathologic knowledge of specific systems in a comparative way, while not being restrained by ethical concerns and huge costs. We will study anatomical phenotypes, diseases and mimic operations without being restricted by time or availability.

The student/researcher will manipulate 3-dimensional anatomical structures up close, from all angles. In addition, dynamic exploration will be supplemented with rich and immediate automated feedback further boosting applicable knowledge, retention, and motivation. The doctor of the future will be equipped

with the knowledge necessary for every day clinical practice, while specialists will improve surgical skills and researchers will be able to deepen and share their knowledge even further. Our 3D-avatar learning concepts are based on grounded educational theories and stimulate active and embodied learning.

We present a working example of a 3-dimensional avatar rat, using Microsoft HoloLens for vivid holographic projection. The student is able to view the avatar from different angles and to show/hide anatomical structures (e.g., bones, muscles, organs, nerves) in an interactive way, using intuitive gestures and voice control. We aim to implement this model within the Dutch Laboratory Animal Science course programs as a replacement to rodent anatomy dissection sessions. Importantly we will investigate how effective the developed tools are in order to reach assigned learning goals.

**Presentation:** Poster

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## Systematic review on the reporting of mouse models for bone healing

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The outcomes of animal experiments can be influenced by a variety of factors, such as animal species, strain, age, housing, husbandry, and appropriate analgesic regimes. Thus, adequate and precise reporting is necessary to obtain reliable and reproducible data. Initiatives such as the ARRIVE guidelines have been enrolled during the last decade to provide a road map and criteria for sufficient reporting. Fracture healing is a finely orchestrated and closely regulated process that relies on the complex interplay between the immune system, the vasculature and bone-forming cells. To understand the sophisticated process of bone regeneration and to develop new therapeutic strategies, small rodents, especially mice, are frequently used in bone healing research. Since many factors might influence the results from those studies, we performed a systematic literature search from 2010 to 2019 to identify studies involving mouse osteotomy models (stable fixation) and evaluate the reporting of general and model-specific experimental details. 254 pre-selected publications were systematically analyzed, showing a high reporting accuracy for the used mouse strain, the age or developmental stage and sex of mice as well as model-specific information on fixation methods and fracturing procedures. However, reporting was more often insuf-



ficient in terms of mouse substrains and genetic backgrounds of genetically modified mice, body weight, hygiene monitoring/immune status of the animal, anesthesia, and analgesia. Raising scientists' awareness of the importance of reporting will improve scientific quality, increase animal welfare, and promote translation to the clinic in the future.

**Presentation:** Poster

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## Innovative animal-free training to stereotaxic neurosurgery

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Stereotaxic neurosurgery in laboratory animals is a demanding technique used in a wide variety of studies in Neurosciences. Allowing to position one or more optical, electrical, or chemical probes, this approach remains indispensable for exploring brain functions. To date however, stereotaxic surgery is not taught as a subject *per se*, but rather passed-on behind closed door in research laboratories, resulting in a variety of practices and success rates. There is therefore a need to harmonize practices and enhance neuroscientists' abilities to explore the brain in a valuable and reproducible way.

Here we introduce an animal-free training on stereotaxic neurosurgery. The teacher/trainee ratio is 1:3 and, as a pre-requisite, trainees validate an online course on elementary concepts such as aseptic techniques, anesthesia and pain management, per-operative animal care, incisions and sutures (Vogt et al., 2011).

The course covers the theoretical background of stereotaxic and focuses on techniques and surgical approaches to optimize spatial precision, while minimizing the risks of irreversible harm to the animal. Anatomy and functional organization of the brain are reminded, with a peculiar attention to the 3-dimensional arrangement of brain blood vessels and ventricles. Hands-on practice includes exercises to acquire an ease in the manipulation of a stereotaxic frame, micropositioners, rulers and verniers. Instead of real animals, trainees use realistic high-resolution simulation devices to measure stereotaxic coordinates of cranial landmarks and entry points, and safely prepare the skull for the insertion and fixation of a probe. Exercises are repeated as needed and the accurate placement of a probe can be checked promptly without the need of histology. Tools and supports are made available for trainees to maintain their skills once back to their laboratory.

Really designed with the 3Rs principle in mind, this course should contribute to promote more reproducible and compassionate approaches in animal research in Neuroscience.

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**Presentation:** Poster

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## Comparing human cell line co-cultures and porcine organoids: Studying (patho)physiological intestinal mechanisms

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Infectious gastrointestinal (GI) diseases are frequently caused by bacterial toxins (e.g., cholera toxin [CTX]), impairing the physiological functions of the intestines. Usually, rodent models are used to study zoonotic GI diseases, although they often fail to mimic species-specific features. To obtain functional intestinal models for studying relevant disease mechanisms without the use of animals, two approaches were chosen: human enterocyte-like Caco-2 cells were co-cultured with human goblet cell-like HT29-MTX cells (Kleiveland, 2015). Additionally, porcine organoids of the jejunum were seeded in a 2D-manner (van der Hee et al., 2018). Using these two models, an *in vitro* study of human and porcine GI barriers focusing on (patho-)physiological aspects was performed.

Epithelial transport properties were investigated using the Ussing chamber system and structural analysis was performed by histochemical staining. As a proof of concept regarding pathophysiology, both models were exposed to CTX in the Ussing chamber.

During cultivation, co-cultures and organoids formed functional barriers of polarized epithelial cells characterized by a high steady-state electrical resistance due to increased expression of tight junction-associated proteins. Incubation of the human co-culture system with CTX resulted in an increase in short-circuit currents as a measure for electrogenic ion transport, corresponding to an increase in apical Cl<sup>-</sup> secretion, confirming



the typical CTX-induced secretory diarrhea in humans. Despite the asymptomatic infection of *Vibrio cholerae* in pigs, CTX induced the same effect in the organoid culture.

Overall, these models have a great potential to serve as physiological models to study interactions of the GI tract with pathogens or pathogen-secreted toxins without the use of animals. Comparison of species-specific characteristics of the human and porcine GI tract can be performed due to the highly comparable responses potentially allowing the identification of differences and similarities of both species.

*Acknowledgement: The project is funded by R2N, Federal State of Lower Saxony.*

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**Presentation:** Poster

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## Engineering 3D vascularized heart tissues using hPSC derived cardiac cells

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The main function of the heart is to pump the blood through the body, via contraction of cardiomyocytes (CMs) that constitute the cardiac tissue (Voorhees et al., 2015). The contraction force produced by the cardiac tissue is a factor that determines the maturity of the CMs. A higher contraction force indicates a higher degree of maturation of CMs, which is more representative of a functional human heart.

Two-dimensional (2D) models do not have the tissue structure and organization of the human heart. In contrast, 3D models have the advantage of resembling tissue organization, function and cell-cell interaction. The current gold standard 3D cardiac *in vitro* model, validated by different scientific groups, is the engineering heart tissues (EHT) (Weinberger et al., 2017).

We have successfully developed a medium-throughput platform that fits in a 12 well plate, where we can produce 36 EHTs using human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs). The contraction force of the 3D cardiac tissues is analyzed with a custom-made software that identifies the displacement of flexible pillars during tissue contraction. Such pillar displacement is converted to force using the Young's modulus of the material. This software automatically goes through the stack of folders with the recordings of the EHTs and provides 2 graphs: force of contraction and contraction speed. For generating cardiac EHTs we used a fluorescent Double Reporter of mRubyII- $\alpha$ -Actinin and GFP-NKX2.5 (DRRAGN) in hPSCs, which allows live recording of sarcomere movement and alignment of cardiac cells.

Here we used this advanced EHT platform for functional analysis of cardiac maturation, by comparing different culture media, electrical stimulation and co-culture of cardiac cells (including hPSC-derived fibroblasts and endothelial cells). These improved culture conditions for cardiac maturation using this EHT platform will be important for a higher predictability regarding human cardiac disease modeling and drug screening.

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## A novel *in silico* framework for *in vitro* model optimization through generalized allometric scaling

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Kleiber's Law (KL) is recognized as a universal law of biology: applicable over 20 orders of magnitude of mass, it states that the basal metabolic rate of an organism scales with its body size according to a quarter-power law (Savage et al., 2004).



The pertinence of KL as a design constraint for developing physiologically relevant *in vitro* models has been highlighted (Ucciferri et al., 2014). Additionally, computational models have been implemented for identifying the size range in which 3D *in vitro* constructs obey KL (Ahluwalia, 2017; Magliaro et al., 2019).

However, KL is generally formulated as a deterministic framework relating average values of the physiological parameters involved. As fluctuations and heterogeneity are inevitable, they should be accounted for in model systems, which should be ideally capable of reflecting the variations found in nature. In this light, we established a novel *in silico* framework for allometric scaling, accounting for fluctuations. Specifically, we implemented finite element modelling for investigating oxygen metabolism in simulated *in vitro* constructs of different sizes, considering stochastic variability for geometrical and biological parameters (i.e., construct radius, kinetic constants). We thus generated joint distributions of construct masses and metabolic rates and developed new statistical tools to test whether and in which size range a generalized formulation for KL (Zaoli et al., 2019) applies.

We found that stochasticity significantly restricted the range of construct sizes complying with KL, implying that many of the current cellular models lack translatability. Our results are under experimental validation by measuring oxygen consumption *in vitro*, comparing the dynamics and the eventually emerging scaling behaviors with those predicted *in silico*.

These studies will enable the definition of robust criteria for improving the physiological relevance of biomimetic models as a key step for designing cell constructs with translational value, paving the way towards alternatives to animal experiments.

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**Presentation:** Poster

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## Evaluation of anti-EGFR induced on- and target-mediated adverse effects in a microfluidic 3D human co-culture model

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The human epidermal growth factor receptor (EGFR) is a key regulator of organ homeostasis mediating physiological cell turnover, proliferation and differentiation and is frequently over-expressed in many tumors (Sigismund et al., 2018). Therefore, EGFR serves as a prime target for tumor therapies. However, its inhibition can induce adverse responses primarily in skin and intestine – the organs with the highest physiological cell turnover (Holcman and Sibilica, 2015).

In this study, we developed a new multi-organ chip-based “safety” assay enabling the simultaneous investigation of efficacy and safety data for anti-EGFR induced cancer therapies (Hübner et al., 2018). The HUMIMIC chip<sup>2</sup> was used to co-culture H292 lung tumor spheroids and a human full-thickness skin equivalent. H292 cells are known to express the target receptor EGFR and are widely used in basic research (Ekert et al., 2014). The culture time was set to five days including a repeated exposure to the anti-EGFR antibody cetuximab. In addition, we compared antibody effects with response data for the tyrosine kinase inhibitor afatinib – a small molecule benchmark drug.

The tumor model showed an aggressive metastatic behavior resulting in the generation of a heterogeneous tissue architecture. Repeated treatment of cetuximab induced an increased expression of pro-apoptotic genes. In addition, antibody-exposed skin equivalents showed an irregular arrangement of keratinocytes accompanied by a complete loss of proliferative keratinocytes in the basal layer. Evaluation of the levels of the proinflammatory chemokines CXCL8 and CXCL10 revealed changes similar to known *in vivo* effects by anti-EGFR treatments.

To the best of our knowledge, no *in vitro* assay exists implementing the requirements to generate efficacy and safety data at the same time. We are confident that it presents a useful tool to be adapted for future efficacy and safety studies of anticancer drugs.

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## Suitability and performance of BioOcular and Epikutis *in vitro* 3D tissue models in China

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Strides have been made in China to develop *in vitro* tissue models for non-animal test methods for evaluating cosmetic products. BioOcular<sup>®</sup> and Epikutis<sup>®</sup> are commercially available 3D tissue models developed by Guangdong (previously Shannxi) BioCell Biotechnology, Co. Ltd. BioOcular<sup>®</sup> is a reconstructed human corneal epithelium (RhCE) model with structure similar to the MatTek EpiOcular<sup>™</sup> model validated for use in OECD TG 492 Eye Irritation Test (EIT). Epikutis<sup>®</sup> is a reconstructed human epidermis (RhE) with similarities to the MatTek EpiDerm<sup>™</sup> and EpiSkin SkinEthic<sup>™</sup> models validated for use in OECD TG 439 Skin Irritation Test (SIT). To examine suitability and performance of the BioOcular<sup>®</sup> model, we partnered with Zhejiang Institute for Food and Drug Control (ZJIFDC) to evaluate ten blinded cosmetic formulations. Duplicate test articles were sent to the Institute for In Vitro Sciences (IIVS) to evaluate in the EpiOcular<sup>™</sup> model. The time to toxicity protocol was used for both sample sets. BioOcular<sup>®</sup> ET50 values had high level of agreement with EpiOcular ET50 values (intraclass correlation coefficient = 0.8444), indicating high interchangeability between the two models. Further BioOcular<sup>®</sup> studies on 21 additional formu-

lations yielded results within expected range based on historical EpiOcular<sup>™</sup> data. To explore the suitability and performance of the Epikutis<sup>®</sup> model, we again partnered with ZJIFDC and IIVS. Three blinded surfactant formulations and a positive matrix control were tested with the time to toxicity protocol. ZJIFDC used China-based Epikutis<sup>®</sup> and SkinEthic<sup>™</sup> models. IIVS used the EpiDerm<sup>™</sup> model. ET50 values for all positive controls were within expected range for all three models. As surfactant concentration increased, ET50 values decreased in all models, indicating a dose-response effect on cell viability. ET50 variabilities were observed between models, which could be due to difference in tissue structure and/or laboratory variability. Overall, our research supports further opportunity for BioOcular<sup>®</sup> and Epikutis<sup>®</sup> models in China.

**Presentation:** Poster

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## Endocrine disruptors – Harmonising with just 1R (Replacement) in mind

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Currently, *in vivo* data is heavily relied on in efforts to identify endocrine disruptors under EU chemical safety regulations. Protocols have been established for identifying endocrine disruptors in plant protection products and biocides and changes to the data requirements for substances under REACH are expected as the European Commission works to harmonise the identification and control of endocrine disruptors across a number of regulatory frameworks. *In vivo* test methods for providing either mechanistic or adverse effect information have a number of serious shortcomings, not least because of the issue of species difference which calls into question the wisdom of using such methods for predicting adverse effects in exposed populations. Moreover, thanks to the cosmetics testing ban, which reflects society's unwillingness to test on animals for the sake of cosmetics products, the harmonisation of regulatory approaches cannot extend to cosmetics so long as the reliance on *in vivo* test methods persists. Nevertheless, full harmonisation across all chemical regulatory frameworks including cosmetics can be realised if the ultimate 1R (Replacement) goal of Directive 2010/63/EU is achieved for the generation of endocrine disruption data. We explore the potential of non-animal methods for providing both mechanistic information and supporting adverse-effect determinations and question the orthodoxy that effective regulation of endocrine active substances necessitates *in vivo* testing.

**Presentation:** Poster



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## Transepithelial electrical resistance as a fast read-out of *in vitro* and *ex vivo* models mimicking the GI-tract inflammation

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The inflammatory bowel disease is a widespread chronic disease of the GI-tract with an increasing number of affected people (Ng et al., 2017). Unfortunately, the previous treatment with immunosuppressants leads to strong side effects and a weakened immune system (Wehkamp et al., 2016). Therefore, the need for new drugs is still high.

The screening of new drugs addressing the inflammatory bowel disease, without the usage of animal experiments, requires robust and reliable models mimicking the *in vivo* situation. We investigated two inflammatory models: First, we developed an *in vitro* co-culture model consisting of Caco-2 cells to simulate the epithelial barrier and monocyte derived macrophages (MDM) as immune cells. Second, an *ex vivo* model using the small intestine of pigs, received as waste from slaughterhouse, in combination with a new electrical measuring system was created. The barrier properties of both models in inflamed and healthy conditions were monitored using TEER-measurements. Additionally, transport studies were performed.

In terms of the co-culture model a reproducible inflammatory state, created by the stimulation of MDM, could be achieved. The TEER values of the Caco-2 cells dropped originating from around 650  $\Omega \cdot \text{cm}^2$  in healthy conditions to around 300  $\Omega \cdot \text{cm}^2$  in inflamed conditions caused by the increased permeability of the epithelial barrier. This could be proven by the increase of Papp-values from 1.63\*10E-7 cm/s to 3.0\*10E-6 cm/s.

Regarding the *ex vivo* model a new set up with four measuring cells could be obtained. The frequency screening from 20 Hz to 200 kHz confirmed that the usage of 1000 Hz as measuring frequency led to representative values. The resistance values for the small intestine depend strongly on the donor and were in a range of 15  $\Omega$  to 100  $\Omega$ . In the next steps the model will be further optimized, and the inflammatory state will be investigated.

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**Presentation:** Poster

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## A novel human material-based platform technology for tissue engineering

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**Introduction:** Human extracellular matrix (ECM) represents the ideal environment for mammalian cells. Placenta is currently classified as clinical waste. A human placenta ECM substrate (hpS) was previously introduced as an alternative to animal material-based products such as Matrigel or fetal calf serum (FCS). The aim of this study was to investigate the potential of hpS as a novel platform technology for tissue engineering.

**Methods:** 2D coating experiments were performed with NIH3T3 fibroblasts or HUVEC, cultivated on either hpS- or Matrigel-coated wells. 3D *in vitro* experiments were performed with HUVEC, or primary colon organoids. FCS substitution experiments were performed with HaCaT, or HepG2, which were cultivated in cell culture media supplemented with either FCS, or hpS. Viability rates were assessed using MTT. Morphologic changes were microscopically analyzed.

**Results:** hpS was well suited as 2D coating material and 3D gel. Viability was highest in NIH3T3 cells growing on 150  $\mu\text{g}/\text{mL}$  hpS coating, whereas the viability rates on Matrigel were significantly lower. HUVEC spontaneously differentiate into cell networks on hpS-coatings with a significantly higher degree of complexity when compared to Matrigel. In 3D *in vitro* studies using a mix of hpS, fibrinogen and HUVEC, or primary colon cells, randomly oriented 3D cell networks, or colon organoids of diameters from 90-240  $\mu\text{m}$ , were formed. Regarding the use of hpS as FCS substitution, HaCaT or HepG2 cells were successfully cultivated in cell culture medium supplemented with hpS instead of FCS. Morphological analysis revealed cell cluster formation only in FCS-supplemented media and slight changes to more stretched morphology were observed in the groups with hpS-supplemented media using HaCaT cells.



**Discussion/Conclusions:** hpS shows high potential as cell culture material for various 2D/3D purposes to substitute animal-derived materials. More experiments have to be performed to assess the full potential of hpS as a platform technology for tissue engineering.

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**Presentation:** Poster

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## Keep it simple, save your money and stick to the preclinical *in vitro* tools for pulmonary drug development

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**Introduction:** Companies asked us to suggest and perform *in vitro* experiments in order to support the development of inhalation drug products. Beside ethical concerns, avoiding animal experiments means reducing development costs. In this context, we want to give a summary of lessons learned and point out new challenges.

**Methods:** Different test strategies were applied to investigate safety and efficacy with *in vitro* models. Complex coculture models, monolayer systems, air-liquid and submerged con-

ditions, primary human cells (immune cells from buffy coat, monocyte derived macrophages, human alveolar macrophages), human mucus & cell lines (hAELVi, Calu-3, A549) were used. Read out was performed by TEER-measurement, ELISA (TNF, IL-6, IL-8), transport studies (sodium fluorescein) and cytotoxicity (MTT and live/dead staining).

**Result:** We recommend submerged culture conditions with Calu-3 and A549 for the first experiments (MTT-assay). They allow for a rough comparison with animal and human data (Metz et al., 2020; Sauer et al., 2013). These first experiments should follow more complex systems and a physical characterization. Air-liquid conditions combined with human cell cultures allow for a relevant safety evaluation (Metz et al., 2018). These data should be combined with the cytokine release of MDM or human alveolar macrophages (Hittinger et al., 2016). Transport studies with hAELVi and Calu-3 can be applied to estimate bioavailability. More details, such as mucus penetration/permeation, can be investigated by additional assays (e.g., transport in Ussing Chamber).

**Outlook:** Method validation of research-based *in vitro* tools and more physiological relevant *in vitro* systems for efficacy evaluation are still required (Hittinger et al., 2017). A human data set for *in vitro in vivo* correlation is needed for the improvement and development of such models.

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**Presentation:** Poster



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## FDA's Alternative Methods Working Group (AMWG) evaluating microphysiological systems for regulatory use

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Advances in systems biology, stem cells, engineered tissues, and mathematical modeling are creating unique opportunities to improve the Food and Drug Administration's (FDA) predictive ability, potentially enhancing our ability to predict risk and efficacy. These advances are expected to help bring FDA-regulated products to market faster or prevent products with increased toxicological risk, including new tobacco products, from reaching the market. Also critical is the potential for these advances to replace, reduce, and/or refine animal testing. The Alternative Methods Working Group's (AMWG) principal goal is to strengthen FDA's long commitment to promoting the development and use of new technologies to better predict human and animal responses to a wide range of substances relevant to FDA's regulatory mission. The AMWG addresses needs and opportunities that typically extend across FDA's product centers and presents current thought on viable ways to foster the development and evaluation of emerging methods and new technologies that will allow FDA to incorporate these methods and technologies into regulatory review of safety and efficacy. In support of FDA's Predictive Toxicology Roadmap, the AMWG's initial case study focuses on the coordination, development, and evaluation of *in vitro* Microphysiological Systems (MPS) for regulatory use beginning with defining agreed-upon terminology for MPS and research/regulatory gaps for which MPS may be useful, identifying partnerships to advance MPS technology, and the development of draft performance criteria for MPS in cooperation with private and government stakeholders. Additionally, FDA has launched a dedicated monthly webinar series on emerging predictive methods to provide an opportunity for developers/users to present new methods and methodologies, providing an open forum between developers and the FDA to discuss translational issues relevant to scientific and regulatory applicability.

**Presentation:** Poster

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## Alternative toxicity testing strategies to advance the 3Rs – A case study on conserved molecular pathways

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Testing chemical compound toxicity is a regulatory requirement, but guidelines typically require extensive testing on large number of animals for approval. Current OECD guidelines are based on traditional methods that require extensive animal experimentation. Organizations face a challenge as the procedure of testing toxicity is time consuming and costly. In addition to that, traditional *in vivo* toxicity testing is often based on fixed set of assumptions or uncertainties, which fail to assist in related regulatory and policy decisions. These methods often lack information on how a toxin might impact genes at the cellular level or its effect in other species. Currently, there is a growing interest in computational methods which can integrate and analyze complex datasets to assist decision making for toxicity testing. Integrating this information would allow generating insight which a single set of experiments might fail to identify. This can then be combined into current tests in regulatory requirements, which might aid in reducing, replacing and refining (3Rs) animal testing. This not only improves the process itself but offers more transparency and validity. By knowing which genes and proteins are conserved across species, the effect of a chemical in a specific species can be non-invasively evaluated. This can facilitate understanding a species susceptibility to a specific class of toxins.

Molecular pathways play an important role in how a specific chemical might affect an organism. Understanding pathway conservation across species (human vs alternative test species) forms the basis for developing reliable pre-screening strategies. This is especially useful for reducing redundancy in toxicity testing of chemicals with large number of analogues.

In this pilot study, we focus on molecular pathways involved in developmental and reproductive toxicity to gain understanding on its conservation across species, with a broader focus on developing methods to advance the 3Rs in toxicity testing.

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## Get-away housing for breeding laboratory rats: Is time away from pups beneficial for rat dam welfare?

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Under natural conditions, rat dams spend increasing amounts of time away from their litter as pups grow older, however rats in laboratories are typically confined in a cage with their pups until weaning. There is evidence that a lack of agency (i.e., the ability to exert control or avoid stressors) may negatively impact animal welfare. We housed rat dams and their litters in cages with or without a loft to allow the dam to spend time away from pups, with the prediction that dams housed without get-away lofts would spend more time passively nursing (i.e., initiated by pups rather than the dam) and experience more negative moods. We assessed dam nursing behavior and anticipatory behavior in response to a food reward (to assess reward sensitivity as an indicator of mood). Results were analyzed using linear mixed models with pup age and treatment as fixed effects with rat identity as a random intercept. Dams without loft access showed increased anticipatory behavior as pups got older, indicative of negative mood ( $24.5 \pm 1.8$  behaviors per minute in week 3 compared to  $18.8 \pm 1.0$  in week 1;  $p = 0.01$ ); there was no effect on dams with a loft ( $p = 0.83$ ). Dams without loft access spent more time passively nursing as pups grew older ( $p = 0.03$ ). At 3 weeks, dams without lofts spent more time nursing ( $59 \pm 2\%$  of time versus  $36 \pm 4\%$  for dams with lofts;  $p = 0.01$ ). Mean time spent in the loft was negatively correlated with mean time spent nursing ( $r = -0.80$ ,  $p < 0.01$ ), suggesting that lofts allowed rats to better control nursing interactions. These findings indicate that an inability to spend time away from pups, especially later in lactation, is associated with increased passive nursing and negative mood; the provision of a get-away loft may improve the welfare of laboratory rat dams.

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## Zebrafish embryo model for chemical-induced cleft palate

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Currently it is necessary to establish alternative testing methods for product safety assessments. However, alternative methods for developmental toxicity tests have not been well developed because of the complicated toxicological responses. Zebrafish early embryos are non-protected animals and one of the promising models for screening of common birth defects owing to conservation between their developmental programs and those of mammals, rapid development and transparency. Although conserved endpoints are necessary for accurate prediction of effects in mammals, little is known about cross-species conservation of teratogenic responses between mammals and fishes. We focused on cleft palate, one of the most frequent birth defects and one for which it is difficult to evaluate chemicals' potential effects using cell culture assays. We investigated a conserved mechanism of cleft palate between mammals and fishes. Zebrafish embryos were exposed to 12 teratogens that induce cleft palate in mammals. Palatal morphology and number of proliferative and apoptotic palatal cells were examined at 96 hpf. By impacting tWnt signaling pathway chemically, we investigated the involvement of canonical Wnt signaling, a key contributor to genetically induced orofacial clefts. All 12 teratogens induced palatal defects in zebrafish embryos, with decreased proliferation and increased apoptosis in the palate. Wnt signaling was inhibited in these zebrafish, and the aberrant phenotypes were rescued at the cellular and molecular levels by Wnt agonist treatment. We identified conserved responses to teratogens between mammals and zebrafish: palatal malformation and regulation of proliferation/apoptosis via the Wnt signaling pathway.

Our results suggest that our zebrafish embryo assay would be a suitable model for assessing chemical-induced cleft palate as well as being a screening tool for prediction of cleft palate in mammals (Narumi et al., 2020). We will confirm the conserved key endpoints by a comprehensive analysis as a next step for accurate prediction of teratogenicity in mammals.

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## Drugs and mucus: Deciphering the interaction mechanisms using a biosimilar mucus model

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Mucus covers the wet epithelia of the human body ensuring protection against air pollutants or pathogens. Drugs administered by oral or pulmonary routes have to overcome the mucosal layer to be absorbed and to exert the therapeutic effect. However, mucus can represent a strong barrier to tackle even for drugs, especially in those pathological conditions where mucus is overproduced (Butnarusu, 2019). Despite the critical role played by mucus on drug absorption, very little is known about the molecular properties that promote the interaction of drugs to mucus, thereby reducing their absorption. In addition, there are no standardized mucus models to be employed in the early drug discovery process for the screening of potential drug candidates.

We have developed a biosimilar mucus model that mimics a pathological mucus (Pacheco, 2019). A natural polysaccharide was used to reproduce the viscoelastic behaviour while the composition was mimicked by adding mucin which is the main glycoprotein forming mucus. The mucus model was coupled to 96-well permeable supports (PAMPA) to recreate mucosal surfaces then was used to study the diffusion of drugs. The mucus model not only represented a physical barrier, but it really behaved as an interactive filter. Different structures related differently to mucus. The diffusion of the majority of the tested compounds was reduced; for some of them, the effect was less pronounced while for a few the diffusion was even enhanced. Multivariate statistical analysis was used to decipher which molecular descriptors play a pivotal role in retention on mucus.

Since drug development is characterized by a high rate of failure, the mucus platform could help to reduce at an early drug discovery stage the number of poor performers that reach pre-clinical trials. Moreover, the model is completely tunable as other mucus components (lipids, DNA, proteins) could be included during the production phase.

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## Better than Matrigel? Alternative cell culture coatings for induced pluripotent stem cell culture and renal podocyte differentiation

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Human induced pluripotent stem cells (iPSC) are widely used in several research areas, including regenerative medicine, disease modelling and *in vitro* toxicology testing. While they hold great promises in terms of reduction and replacement of animal experiments in the future, their culture requires the use of an extracellular matrix (ECM), which is most often Matrigel, a product produced in animals. Matrigel is produced in mice, by inducing them with sarcoma tumors. The replacement of Matrigel with animal free products is therefore desired for both, ethical and scientific reasons, including better standardization conditions.

Here, we carefully evaluated and compared existing alternative products, including human recombinant Vitronectin, Laminin-521 and Laminin-511, with a major focus on iPSC quality and stability. Furthermore, we tested more cost-effective coatings by employing human cell lines to harvest ECM. Pluripotent markers, including Nanog and Oct4 and the capacity of cells to form embryoid bodies were analyzed via High Content Imaging in 3 iPSC lines cultured for several passages on alternative coatings. Furthermore, transcriptomics (TempO-Seq) and shallow DNA sequencing (for copy number variations) were performed. In addition, differentiation of iPSC into renal podocytes (as described by Rauch et al., 2018) were tested on alternatives coating and compared to Matrigel. iPSC-derived podocytes were stained with podocyte-specific markers, including synaptopodin and WT1.

Our results show that alternative coatings are well suited for both, maintenance of undifferentiated iPSC as well as differentiation of iPSC into renal podocytes.

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**Presentation:** Posters

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## Effects of testing moment on behavior and cognition in the laboratory mouse

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The daily light-dark cycles allow the synchronization of behavioral and physiological processes to the external environment. Light is the most important environmental cue or zeitgeber that coordinates many aspects such as activity, maintenance behaviors, alertness, body temperature, hormonal regulation or long-term potentiation (Fisk et al., 2018; Peirson et al., 2018). Mice are among the main animals used in behavioral neuroscience and preclinical research laboratories. Although nocturnal, they are generally tested during day (i.e., during their resting phase). Even if convenient for the experimenter, a perturbation of the sleep-wake cycle such as manipulations during day can generate some stress to the animal, produce few reliable data and may lead to negative consequences for health, physiology, behavior and cognition (Hawkins et al., 2018; Peirson et al., 2018). Moreover, several cognition studies about memory, learning, cognitive flexibility or attention have shown that mice performed better when tested during their active phase (i.e., the night) and, recently, a lack of data related to the effect of testing moment on behavior was highlighted (Hawkins et al., 2018; Peirson et al., 2018). Thereby, the testing moment might be a predominant variable affecting animal behavior and therefore all the inferences we make about cognitive processes.

In this study, we focused on an anxiety test (the Elevated plus Maze, EPM) and a memory test (the Object Recognition Test, ORT). We tested 4 different testing moment, two in the light phase and two in the dark phase, to determine if there is a more appropriate testing moment for animal experimentation where mice show the less anxiety and the best performance. Then, we hypothesize that mice tested during their active period will perform at least as good as mice tested in their rest period, arguing a change in laboratory practice.

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## The use of animal-derived products in *in vitro* methods for skin sensitization testing

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*In vitro* methods are of utmost importance in the replacement of animals for cosmetic testing. However, most of the current cell culture and associated techniques for skin sensitization prediction apply several animal derived products (Marigliani et al., 2019a). Some of these products hold serious technical and ethical issues, which is the case for fetal bovine serum (FBS), a cell culture media supplement known to exhibit batch-to-batch variations and contamination by microorganisms. FBS is extracted from bovine fetus blood, collected by cardiac puncture without anesthesia (Van der Valk et al., 2004). A systematic review of *in vitro* methods for skin sensitization testing showed that 78% of the 83 analyzed methods (identified among 156 articles) use FBS. Inclusion of animal sera was found in 100% of the methods used to assess keratinocyte activation and in 94% of those used to assess dendritic cell activation, while bovine serum albumin was clearly mentioned by 25% and 62% of these, respectively. A total of 92% of the methods to assess dendritic cell activation and 100% of those to assess T-cell activation mentioned the use of animal-derived, monoclonal/polyclonal antibodies (Marigliani et al., 2019a). However, animal-free alternatives are emerging and there are successful examples of recent efforts to adapt cells to chemically defined media (Marigliani et al., 2019b) and to adapt validated methods to animal-product-free conditions (Belot et al., 2017; Edwards et al., 2018). As there are non-animal alternatives to many of animal products and as the social demand is for cruelty free cosmetics, more effort should be made not only to replace animals in cosmetics safety assessment, but also to replace animal-derived products in validated *in vitro* methods and to keep them out of the development of new ones, in order to have technically and ethically better methods for cosmetic testing.

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## Students' and researchers' attitudes to the 3Rs at the Karolinska Institutet – The importance of education

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Besides ethics and science, attitude and behavior are key components in 3R development. Researchers, as main users of research animals and animal-free models, have opportunities and responsibilities to contribute to 3R development. Students is an important target group for 3R education as next generation of scientists and specialists in academia, authorities and private industry.

We have been following students in the Global Master's Programme in Toxicology at Karolinska Institutet, in which the 3Rs are integrated into all courses. Students' attitudes were compared with researchers at Karolinska Institutet in a 3R-survey using the response alternatives Strongly disagree, Disagree, Neither/Nor, Agree, Strongly Agree. Respondents were divided into three groups: students, junior researchers and senior researchers (N = 44, 63, and 44, respectively).

When asked about usefulness of the 3Rs when defining research question and reporting data, the majority agreed or strongly agreed (69-71%, no differences between the groups). All three groups agreed to a higher extent (79-100%) to usefulness related to animal handling, housing and euthanasia, students even more than the researchers (95 to 100%,  $p < 0.05$ ), indicating high engagement in Refinement. The vast majority in all groups agreed that stressed animals yield less valid results (93, 95, 91%). Stu-

dents agreed that 3R results in increased scientific quality more than junior and senior researchers, respectively (Replacement 55, 23, and 28%; Reduction 52, 24, and 28%; Refinement 93, 79, and 72%,  $p < 0.05$ ).

In general, all respondents showed an equal positive attitude towards the 3Rs, especially Refinement. Observed differences indicate that students in some cases are more positive. This might be due to differences in responsibilities/experience, and that younger generations have more recent and updated knowledge in animal ethics and behavior and higher confidence in computer and cell models. This will be further explored, e.g., by filming the students learning in a documentary during 2020.

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## Characterization of chemical substances using cytochrome P450 inhibition data

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To establish alternatives to animal testing for chemical safety assessment, *in silico* methods, such as (quantitative) structure-activity relationship and category approaches/read-across, are well studied. In these methods, molecular descriptors are often used to describe chemical and structural properties of chemical substances. However, it remains very difficult to establish such an *in silico* method using molecular descriptors for some toxicity, such as repeated-dose toxicity. This is partly because some chemicals exhibit their toxicity after metabolic activation in the body. Cytochrome P450 (P450) is the group of enzymes with different substrate specificity and involved in both detoxification and metabolic activation of chemical substances. In this study, we have investigated possible use of P450 inhibition data as a tool to characterize and classify chemical substances. Inhibiting ability of 220 test compounds against 10 human forms and 7 rat forms of P450s (family 1-3) were determined using recombinant enzymes (Supersomes; Corning) and P450-Glo assays with chemiluminescent substrates (Promega). A total of 148 (67%) and 201 (91%) compounds inhibited at least one P450 by  $> 15\%$  and  $> 10\%$ , respectively, at 0.1, 1.0 or 10  $\mu\text{M}$ . Among the P450s tested, human CYP1A1, CYP1B1, CYP2C19, CYP3A4 and rat CYP1A1 and CYP2B1 were highly inhibited while rat CYP2D1 and CYP2E1 were less inhibited. Pearson's correlation analyses of the P450 inhibition profiles demonstrated no significant correlation for most of the combinations except the human and rat or-



thologs and the same subfamily members, as expected from the P450s' substrate specificity. When the test compounds were divided into 14 groups by hierarchical clustering using the inhibition data, most of the groups consisted of structurally diverse compounds although nearly identical compounds tended to be in the same group. These results suggest that P450 inhibition profiles obtained by high-throughput *in vitro* assays can be used as novel biological descriptors for characterizing chemical substances.

**Presentation:** Posters

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## Assessing the uptake of research dog adoption

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In the U.S., around 60,000 dogs are used annually for research and testing. Many will undergo terminal procedures, whereby their tissues are collected for histological analysis and thus the dogs are euthanized, denied any chance of a normal healthy life outside the laboratory. However, U.S. legislation urging the adoption of companion animals used in research was started in 2014 in Minnesota, with 13 states passing similar bills to date, and federal legislation under consideration. Additionally, the Food and Drug Administration (FDA) recently announced its intention to permit the adoption of healthy animals once they have been used in experiments (Bucchino, 2020), following similar policies adopted by various other federal agencies. The species included in laws and policies varies and as yet, no U.S. law requires reporting of the number of animals rehomed.

We set out to assess the impact of formal legislation on dog adoption, specifically requesting the number of dogs adopted following their use in research and testing, and organization policies on the adoption of dogs. Here, we will report on the results of public information requests made to government-funded institutions in order to ascertain levels of adoption. Analysis of U.S. Department of Agriculture (USDA) reports indicates that the bulk of dog use is in private laboratories, where we have no legally mandated access to data. Despite the lack of legal obligation for private companies to disclose requested information, we also reached out to those companies using dogs to let them voluntarily provide their data on adoption numbers and policies.

Based on these responses, here we make recommendations to enhance adoption rates, including the incorporation of reporting requirements into new legislation and existing policies, which will increase adherence to the intent of legislation and policies for rehoming of dogs out of laboratories.

## Reference

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**Presentation:** Posters

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## Are dogs still needed in safety testing of pesticides?

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When registering a new pesticide active ingredient (AI), sub-chronic 90-day oral toxicity studies, performed both on rats and dogs, are standard testing requirements and are intended to be representative of shorter-term incidental pesticide exposures. To determine how often the 90-day dog study was selected as the point of departure for assessing incidental risk, human health risk assessments (RAs) for 137 pesticide AIs registered by the U.S. Environmental Protection Agency within the last 22 years were reviewed. The dog study was used for the point of departure in 36 of these RAs because the dog NOAEL (No Observable Adverse Effect Level) was the lowest of all the shorter duration tests. In nine of the 36 cases, the lower NOAELs could be attributed to dose spacing when compared to the rat 90-day study, meaning the dog was selected as an artifact of dose setting not because it was the most sensitive species. When the principles of allometric body weight scaling based on metabolic rate (Kleiber, 1932, 1947), which can account for larger species showing effects at lower doses than smaller species (Schneider et al., 2004), were applied, dog and rat NOAELs were at "equipotent" doses for 23 of the 36 AIs. Only 13 AIs remained for which dose spacing and/or body weight scaling could not account for the dog appearing to be more sensitive, although for some of these AIs, studies with comparable NOAELs were available. Overall, impacts to RA were minimal for nearly all AIs without the dog study, but in a few cases identification of important hazards may have been missed. The results of this analysis suggest that in most cases the 90-day dog study does not add materially to pesticide risk decisions and provide a basis for developing criteria to waive it.

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**Presentation:** Posters



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## Use of the full thickness model, T-Skin™, to investigate the effects of UVA and UVB on skin and photoprotective effects of vitamin C

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The skin represents a major barrier to penetration of chemicals into the systemic circulation. It is also subject to damaging effects from environmental factors such as UV radiations. The safety of topically applied pharmaceuticals and cosmetics, as well as topically exposed environmental chemicals, requires a robust and predictive skin model. T-Skin™ is a reconstructed full-thickness model with a well stratified, differentiated and self-renewing epidermis and a dermal compartment composed of functional fibroblasts embedded in a matrix of collagen I (1). We have used this 3D model as a reproducible, *in-vivo*-like, and predictive human skin model to (a) characterize the effects of UVA1 and Solar Sun radiation UVA1 + UVB (UVSSR) exposure on skin tissue and (b) to investigate the protective effects of vitamin C against UVA1 radiation damage. Endpoints measured were tissue viability, histology, fibroblast number and cytokine and chemokine release. Classical photoaging responses of human skin due to UVA1 exposure (Biological Efficient Dose) were observed i.e., a viability decrease mainly located in the dermis associated with a loss of a dermal fibroblasts disappearance, a metalloproteinase-1 pro-inflammatory mediators releases. UVSSR caused damages to both epidermis and dermis parts, and a more extensive release of inflammatory mediators than UVA1 exposure. Vitamin C protected against UVA1 induced damages, evident at level of dermal damage (preventing a loss of viability and the number of fibroblasts in the dermis and the attenuation of metalloproteinase-1 release), as well reducing the release of IL-1a and IL-8. This study shows that the T-Skin™ model mimics the main responses to UV radiation and supports its use as screening tool to develop new UVA1-protective ingredients.

### Reference

Bataillon, M., Lelièvre, D., Chapuis, A. et al. (2019). Characterization of a new reconstructed full thickness skin model, T-Skin™, and its application for investigations of anti-aging compounds. *Int J Mol Sci* 20, 2240. doi:10.3390/ijms20092240

**Presentation:** Posters

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## Make people better scientists in the lab: Alvertox vision

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Alvertox Academy connects international experts to provide hands-on-training (HOT) in human-relevant alternative methods and technologies for toxicologists of all levels of experience, from entry level technician to laboratory or department manager. The participants will become familiar with new technologies and their critical steps. A HOT is a perfect way to quickly approach a method. The training allows not only to understand methods that researchers want to set up, but also the data analysis and interpretation that could be a critical step when generating results. In the past three years, Alvertox Academy has organized more than 30 HOT with a format allowing a detailed and practical description of the methods (20% lectures and 80% HOT or case studies throughout two days). With a maximum of 15-20 participants, divided in small groups for the practical component of the training, this format allows networking and connects experts to people that will daily use their method. Focused on alternatives to animal testing, the topics covered by our trainings are *in silico* methods (endocrine disrupting compounds, *in silico* models for cosmetics) and *in vitro* methods (lung inhalation, skin sensitization, hepatotoxicity and more). Promoting education and training brings improvement in scientists' day-to-day work and can also have a positive impact on the general scientific community. Participating in our trainings will improve your skills for a specific method, showing each method's limitations, and provide you with the capacity to challenge the tests and interpret data. Alvertox Academy follows also the vision to MAKE scientists BETTER citizens, by offering a Skills4Science training for young researchers. The primary focus of "Skills4Science" is to tackle topics that do not emerge during conventional scientific congresses and to empower in particular young researchers on understanding social media influence on researchers' activity, gender inequality, scientometrics and scientific collaboration.

### Reference

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**Presentation:** Posters



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## Virtual class on alternative methods in toxicology

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In the second semester of the AY 2019/2020, started a pilot on-line version of the course “Alternative Methods in Toxicology”, a mandatory module in the master curriculum Veterinary Biotechnology Sciences, first year, in the Università degli Studi di Milano.

The module, 4 credits/30 hours, is focused on 3R concept, alternative test methods and new approach in toxicology, stand-alone methodologies, Integrated Testing Strategy, validation. The module is in English and is designed to provide 3 hours per week, 18 hours lectures and 12 hours practical activity.

Students are weekly informed by the teacher on the scheduled lectures and course activities thorough a notice-board present on an on-line platform (<https://fcalonimat.ariel.ctu.unimi.it/v5/home/Default.aspx>), specifically dedicated. The students are invited to follow the video lessons provided according to a scheme uploaded on the platform, and subsequently to take part to a video conferencing in a Virtual Class for a synchronous action with the teacher, entering with a code, where an open and dynamic discussion starts on the topic of the day. Other didactic material, i.e., articles, slides, videos, is also available for the students and shared during the Virtual Class. Corner, forum and meeting are planned, and external experts are also invited to attend. The Virtual Class is also the place where students give suggestions on possible implementation and strategies on alternative methods and identify some topic priorities.

The Virtual Class seems to evoke an evident and stimulating interest from the student perspective, demonstrated by the continuous and active participation.

At the end of the course will be required to the student to fill a questionnaire.

*Acknowledgment: We acknowledge the students of the first year of Veterinary Biotechnology Sciences Course, Università degli Studi di Milano AY 2019/2020*

**Presentation:** Posters

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## Nearly 200 million animals used in medical research worldwide

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The use of live animals in experiments remains controversial. However, there is a lack of reliable data on the scale of animal testing. Almost 80% of the world's animal using countries do not publish the numbers they use.

In 2005 Cruelty Free International set about trying to provide an overall estimate for the worldwide use of animals in harmful research. We could find only 37 countries, mostly European, which provided annual statistics. We created a simple regression model, using publication of animal-based research as a proxy for the number of animals, to extrapolate figures for non-reporting countries. For the year 2005 we estimated approximately 115 million animals per year were used worldwide.

Ten years later we set about to update the statistic (Taylor and Alvarez, 2019). Unfortunately, the number of reporting countries had not changed and this time the estimate for 2015 was 192 million animals per year. Most of the increase could be explained by increased science publications from Asian countries- meaning that our estimates for their use of animals in research are probably now more accurate.

We estimate that China is the greatest user of animals in experiments (20.5 million), followed by Japan (15 million) and the United States (14.6 million). The United Kingdom, Germany and France are also in the top ten, being the largest users in Europe with around two million animals each. We also estimate there were over 200,000 tests on dogs and nearly 160,000 tests on monkeys in 2015.

We hope our new estimate will encourage greater efforts to replace and reduce animal use whilst also acting as the baseline from which to measure success.

### Reference

Taylor, K. and Alvarez, L. R.(2019). An estimate of the number of animals used for scientific purposes worldwide in 2015. *Altern Lab Anim* 47, 196-213. doi:10.1177/0261192919899853

**Presentation:** Posters



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## The prevalence of gavage incidents in regulatory toxicity studies

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Gavage is the preferred technique in many regulatory and experimental animal studies requiring the oral ingestion of test substances. It is well established that gavage is stressful and that occasional deaths can occur due to “gavage incidents”. Less well appreciated is the prevalence of signs of “gavage-related reflux” (GRR), which include respiratory distress, rooting or eating bedding, excessive salivation and nasal discharge, throat or lung injuries which can also lead to death. There has been no comprehensive survey of the prevalence of gavage-related incidents in regulatory toxicology studies.

In this study, we reviewed the robust study summaries of 333 repeated dose toxicity studies published in the EU REACH chemicals database. 13% of studies reported that there had been at least one death as a result of the gavage procedure; range 1-22 animals per study. A further 11% studies reported signs that the study report considered were consistent with gavage injury or reflux; an additional 16% reported signs including deaths that were consistent with GRR or gavage injury but were not reported as such and a further 14% reported excessive salivation, a key sign of GRR. In total over half of the studies were affected by deaths or signs of GRR in some of the animals.

Deaths due to gavage incidents were more likely to occur in treatment groups suggesting that the properties of the substance can play a role in GRR and/or difficulty in performing the procedure. Signs of GRR or deaths were more likely to occur with corrosive substances. Urgent review of the use of the gavage procedure is needed on both scientific and welfare grounds.

**Presentation:** Posters

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## Completing the replacement process, why do some animal tests persist?

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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 al-

ternative methods. Building on over 20 years of experience with this specific issue, over the last 5 years Cruelty Free International has been closely tracking a number of animal tests to identify the scale and cause of this phenomenon.

In an effort to draw attention to the issue, we produced the Replace Animal Tests (RAT) list which highlights ten common animal tests with accepted non animal alternatives or where the scientific consensus is heavily towards redundancy. The list includes the rabbit pyrogen, skin irritation and eye irritation test, the guinea pig skin sensitisation test and the mouse botulinum toxin, shellfish toxin and skin sensitisation assays. There are others of course. The total numbers of animals that this affects could be in excess of one million in Europe alone.

In this presentation we provide statistical evidence that these tests are still being conducted and open for discussion the reasons why this may be. Some reasons are well understood, such as the desire for international harmonisation before absolute deletion, others could be due to a lack of monitoring and enforcement. It is imperative that these animals are not forgotten, and that the replacement process is truly completed for both scientific and moral reasons.

**Presentation:** Posters

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## The monocyte activation test as alternative to the rabbit pyrogen test: Choosing the optimal serum source for the assay

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The monocyte activation test (MAT) is used to detect pyrogens in pharmaceutical products and serves as a replacement of the rabbit pyrogen test (Ph. Eur. chapter 2.6.30, 2017). The peripheral blood mononuclear cell (PBMC)-based MAT assay requires the addition of serum to the cell culture medium which could be fetal bovine serum (FBS) or human serum (HS). Since the capacity to detect non-endotoxin pyrogens (NEPs) in a sensitive manner is an important strength of the MAT compared to, e.g., the bacterial endotoxin test (BET), the performance of the MAT using FBS and HS as serum source was investigated using endotoxin and several NEPs (flagellin, peptidoglycan, R848, heat-killed *Staphylococcus aureus* and Pam3CSK4). The MAT, using a cryopreserved pool of PBMC's from 4 donors, was more sensi-



tive for endotoxin when FBS was used as serum source, however for most NEPs the MAT was more sensitive when performed in HS. Heat-inactivation of FBS had minor effects on the performance of the MAT for endotoxin, but not for the NEPs. Interestingly, heat-inactivation of HS led to an almost complete loss of reactivity towards endotoxin and reduced reactivity in the response towards HKSA and PGN, while having minor or no effects on the responses towards R848, flagellin and Pam3CSK4. Therefore, to guarantee optimal performance of MAT heat-inactivated serum should be avoided. Moreover, product testing of a human blood-derived product in MAT using HS was beneficial since endotoxin spike recoveries were improved. This product is therefore currently batch released with the HS-based MAT assay. Overall, The HS-based MAT appears to be the first choice to replace the rabbit pyrogen test while in some cases the FBS-based MAT may be favored.

#### Reference

European Pharmacopoeia, 10<sup>th</sup> edition. EDQM, Council of Europe, chapter 2.6.30 (07/2017)

#### Presentation: Posters

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### Guidance on dose-setting in repeated-dose toxicity studies; outcome of an ECETOC Task Force

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The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) has established a Task Force with the objective to provide guidance on dose-setting to support appropriate design and interpretation of toxicity studies. Recent regulatory opinions mainly in the EU, and driven by classification

needs, have suggested that there should be a systematic move to increase the dose levels used in repeat dose toxicity studies, combined with a resistance to the use of kinetics to inform dose level selection. Beside animal welfare consideration, inappropriate dose setting hinders our ability to correctly estimate human risk while overestimating hazard, identifying the urgent need for science-based guidance.

In the development of the guidance, we proposed practices that reflect the current state of the sciences, considering the following elements. A) Discussion of the dilemma of studies expected to fulfil a dual purpose of providing information for risk assessment as well as for hazard characterization, indicated that human-relevant and exposure-led information should be preferentially used in dose selection where appropriate. B) The critical review of the basis for using a Maximally Tolerated Dose led to a consensus that there is no direct link between increasing doses beyond this value and better protection of human health, neither for serving classification needs or for risk assessment/risk benefit analysis. C) Existing practices in dose-setting across industrial sectors and regulations have been reviewed, including the value of range-finding *in vivo* studies. The review included dose selection parameters indicated in current (primarily OECD) test guidelines, some improved assessment of maternal toxicity in reproductive studies and preparation of sector specific recommendations (including pharmaceuticals, chemicals, agrochemicals, biocide, food) D) Guidance for use of toxicokinetic and pharmacodynamic evidence in dose setting was prepared reviewing approaches and examples, confirming that testing in non-linear kinetic ranges correspond to exceedance of Maximum Tolerated Doses.

#### Presentation: Posters

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### Development of functional 3D cardiovascular construct

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To mimic human heart, *in vitro* cardiac model should accurately recapitulate the 3D structure and vasculature of healthy and diseased cardiac tissue. Animal models and 2D cell cultures fail to capture human cardiac physiology due to species differences in electrophysiology, beat rate and in 3D microenvironment. We have previously shown that vascular structures support the maturation of pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) in 2D culture (Vuorenmaa et al., 2017) and that hypox-



ia affects the structure and function of the cardiomyocytes (Häkli et al., 2021). Now, we establish 3D cardiac construct with vascular structures resembling human heart in healthy and in ischemic conditions.

To form 3D construct, novel hydrazone crosslinked gelatin-gellan gum hydrogel was used. For this, carbodihydrazide (CDH) Gelatin A 60 mg/ml and gellan gum 40 mg/ml (GG, Gelzan CM Gelrite) (Koivisto et al., 2019) were dissolved in Dulbecco's modified eagle medium. First, vascular structures were formed by self-assembly of human adipose stem cells and GFP expressing human umbilical vein endothelial cells (Cellworks) in the hydrogel. After 7 days, hPSC-CMs were seeded either embedded in the hydrogel or as a monolayer on top of the 3D vascular structures. Tubule formation, cellular morphology, orientation and cardiac functionality were characterized after 9 to 15 days of co-culture.

The results showed the stability of gelatin-gellan gum hydrogel for 7-15 days. The hydrogel supports formation of alpha smooth muscle-positive cell network and modest vasculature. In 3D co-culture, the cardiomyocytes show elongated morphology and aligned orientation. Contractility of the 3D construct is more synchronous and visually stronger compared to cardiomyocyte monocultures.

The 3D environment with vascular structures improved the functionality of the hPSC-CMs exhibiting features that mimic the complex *in vivo* conditions. The 3D cardiovascular model can be used to study mechanisms of myocardial ischemia with our custom-built hypoxia chamber (Häkli et al., 2021).

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**Presentation:** Posters

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## ***In vitro* modeling of gastrointestinal exposure and response to engineered nanomaterials**

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There is a growing number of applications for various engineered nanoparticles (ENP), however, their impact on human health and various organs is poorly understood. In this study we utilized a 3D reconstructed human intestinal microtissues, Epi-Intestinal tissue model, to develop an *in vitro* system for assessment of toxicological profiles of ingested nanomaterials. The tissues were exposed to various concentrations of three types of nanoparticles: copper (II) oxide (CuO) (50 nm in size), zinc oxide (ZnO, 35-45 nm in size), and titanium oxide (TiO<sub>2</sub>, 40 nm in size). Sonicated nanoparticles were resuspended in 40 µl of buffer and applied apically onto the tissues for 4 hours. Following application, the tissues were washed and incubated for additional 24 hours in standard medium. Subsequently the barrier integrity (TEER) and viability (MTT) were determined for each tissue. In addition, medium was collected to determine levels of selected pro-inflammatory cytokines released following the nanoparticle exposure. Using IC15 (concentration of nanoparticles that reduces barrier function or tissue viability by 15%) as a cut off, we observed a dose response reduction of barrier integrity and tissue viability for CuO and ZnO. On the other hand, the titanium oxide did not induce toxic effects even at the highest concentration. Similar observation was detected through the cytokine production – we have seen a dose-dependent increase of interleukin 8 (IL-8) in tissues exposed to CuO and ZnO. Overall, we have shown that the TEER measurement is very reproducible and more sensitive endpoint than MTT. In conclusion, these results suggest that reconstructed small intestine tissues might become a sensitive tool not only for determining the toxicity of ingested nanoparticles, but also for further studying interactions of nanoparticles with host gastrointestinal system.

**Presentation:** Posters

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## Development of an “agitated” *in-vitro* test for glass fiber dissolution

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Mineral (glass and stone) wool is one of the most used insulation materials, due to its outstanding effectiveness, but also because of extensive and robust studies supporting the fact that they are safe to use when standard safe work practices are followed. Specific protocols to characterize fiber biopersistence have been defined by European authorities (Bernstein et al., 1999). Tested according to one of these protocols, the fibers have to demonstrate a low biopersistence to not be classified as carcinogenic under the EU CLP Regulation (Note Q, Regulation (EC) n°1272/2008). Mineral wool manufacturers, such as Saint-Gobain, need to reduce as much as possible the number of these *in-vivo* tests, as they raise ethical issues and are costly and time-consuming. Thus, the development of *in-vitro* tests reliable, quicker, cheaper and predictive of *in-vivo* biopersistence is required.

EURIMA (EUROpean Insulation Manufacturers Association) (Sebastian et al., 2002) aims to develop an acellular *in-vitro* test, in which fibers dissolution in a flow-through system is followed by chemical analysis of the solutions. Saint-Gobain has a long experience on dissolution tests (de Meringo et al., 1994; Thelohan et al., 1994; Guldborg et al., 2000) and has decided to develop an acellular *in-vitro* test in a different way, with a closed system in which the dissolution fluid is agitated but not circulating.

In this work, the Saint-Gobain *in-vitro* test is presented; the parameters impacting glass wool and stone wool dissolution, such as the fluid composition and pH, are studied; and differences between “circulating” and “agitated” *in-vitro* dissolution tests are discussed. The “agitated” fluid allows to reduce the test duration and to maintain a constant pH during experiment, which increases the reproducibility. This test is still under development as it also displays limits. Further efforts will be needed to obtain a robust predictive tool.

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**Presentation:** Posters

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## IPAM, the Italian Platform on Alternative Methods

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National Platforms for alternative methods were created about twenty years ago with the aim to promote and inform about alternative methods to speed up their regulatory acceptance. To achieve these goals effectively and incisively, collaboration between the following four areas was considered as a priority: Research – Industry – Government Institutions – Associations for animal protection.

The Italian Platform on Alternative Methods (IPAM), in full compliance with this approach, pursues at: i) promoting research and information on alternative methods in animal experimentation in Italy, ii) building synergies to accelerate development and acceptance of alternative methods in basic, applied, and regulatory research. Within this framework, IPAM regularly organizes national and international events and meetings (Rovida et al., 2013; Nagy et al., 2016; Caloni et al., 2018) aimed at different stakeholders (students, researchers, general public). Among the most recent, the exhibition “Science & Consciousness, a journey inside the 3Rs” a didactic itinerary on alternatives in animal experimentation which was hosted by several Italian universities and research institutes, and the IPAM-ecopa symposium 2019 on “Non Animal Methodologies (NAMs): research, testing, assessment and applications”, recently co-organized with ecopa (European consensus-platform for alternatives) (Lorenzetti et al., 2020).

Moreover, since 2007, the IPAM-Farmindustria award is assigned every two years to a young researcher author of a paper and/or a thesis degree relevant to the application of 3Rs Principle in pharmacological research. IPAM also actively dialogues with national and international regulatory bodies and its members frequently share their expertise in training events organized by university, industries, and public entities.



Finally, the IPAM's website ([www.ipamitalia.org](http://www.ipamitalia.org)) and Facebook social ([www.facebook.com/IPAMITALIA](https://www.facebook.com/IPAMITALIA)) represent an important point of reference of information, updates, and discussion for anyone who is interested on the 3R Principle, alternative methods and their applications in science.

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### Presentation: Posters

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## A novel *in silico* tool for dose assessment in cell monolayer nanotoxicology

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Due to the widespread use of Engineered Nanomaterials (ENMs), *in vitro* ENM dose assessment appears crucial in nanotoxicology (Sohal et al., 2018). In this context, *in silico* modelling provides an outstanding tool for predicting the amount of ENMs delivered to biological tissues in specific configurations (Poli et al., 2020). In literature, two computational models have emerged regarding ENM dosimetry: the DG (DeLoid et al., 2015) and the ISD3 model (Thomas et al., 2018), implementing the 1D dynamics of nanoparticles through a protein-enriched culture medium with a cell monolayer on the bottom.

In this study, we firstly integrated these models within a Graphical User Interface (GUI) purposely developed in Matlab, enabling to identify the most suitable one for a specific application. Then, we exploited it for insoluble ENM (i.e., CeO<sub>2</sub>, TiO<sub>2</sub>, and BaSO<sub>4</sub>) dosimetry. The predictions of the GUI in terms of middle height concentration profiles over time for such ENMs were successfully validated (R<sup>2</sup> > 0.75) by fitting them on the corresponding experimental profiles, measured using a gelatin

coating for mimicking the totally sticky bottom condition implemented *in silico*.

Moreover, since nanoparticle uptake by cells (i.e., the bottom stickiness) is an ENM-specific feature, we exploited a plasma-spectrometer for assessing the cumulative dose internalized in HepG2 cell monolayers after different time of exposure to the three insoluble ENMs. The results revealed significantly different adsorption profiles, allowing to accordingly tune the stickiness parameter of the models for each ENM and thus to improve the capability of the GUI in determining the cumulative effective dose reaching cells as a function of the initially administered dose.

Further *in vitro* testing is ongoing for relating effective dose predictions with corresponding biological effects, refining the dose-response characterization, and establishing the proposed tool as the basis for a reliable alternative to expensive and ethically sensitive animal experiments.

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## TargetTri: Safety assessment and de-risking of novel drug targets

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Assessing safety liabilities of (exploratory) drug targets in the early preclinical phase of development is an important aspect of managing the drug discovery pipeline. On the one hand, toxicity information is imperative for the de-risking of therapeutic targets that are progressing through the development pipeline. On the other hand, safety considerations can aid in the prioritization



and selection of exploratory drug targets. Both contribute to the reduction of attrition rates that have hampered the launch of new drugs in recent years.

TNO, a non-profit research organization, is developing the target triaging platform “TargetTri” together with pharmaceutical partners. By combining data mining, text mining, and systems biology/network analysis, this web-based platform offers insight in various aspects of target-related toxicities in separate views. These provide for example information on diseases, biological (adverse) effects, SNPs, transgenic models, pharmacological tools, clinical data and expression profiles and subsequent risk mitigation strategies.

The TargetTri platform has been applied to analyze and de-risk Oncostatin M (OSM), identified as a potential therapeutic target for cardiovascular disease by the CarTarDis consortium. Identified major risks concerned anemia and increased susceptibility to infection based on clinical and preclinical data. Reduction of myocardial remodeling and angiogenesis may also be of concern for the atherosclerotic patients based mainly on preclinical studies. The occurrence of such effects needs to be evaluated in subsequent de-risking assays supported by TargetTri.

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## Subacute 28-day respiratory toxicity assay using an *in vitro* human airway model

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Determining the subacute (28-day) respiratory toxicity potential is an important component of establishing safety profile of chemicals and consumer products. Here we describe our efforts to develop an alternative, non-animal method for determining subacute respiratory toxicity using the EpiAirway™ *in vitro* human airway model. The first stage consisted of determination of acute toxicity. The EpiAirway tissues were exposed for three hours to four concentrations of test chemicals via apical application using either aqueous or corn oil vehicles. After rinsing, the tissues were incubated for additional 21 hours in standard cultivation conditions. Barrier integrity (determined by measuring transepithelial electrical resistance (TEER)) and viability (MTT assay) were determined and an IC75 concentration (concentration required to reduce the endpoint value to 75% of vehicle exposed controls)

was determined. Based on the determined acute IC75 value, Epi-Airway tissues were exposed to additional serial dilutions of the test chemicals, using the IC75 as the baseline dose. Every Monday, Wednesday and Friday the tissues were apically exposed for 3 hours and subsequently washed. TEER was measured before each application. Experiments continued for at least 30 days to determine no-observed-adverse-effect level (NOAEL) doses. Rank ordering of NOAEL levels obtained for 8 chemicals was as follows: formaldehyde << butyl amine < oxalic acid << vinyl acetate < morpholine < methyl methacrylate << dimethylacetamide < ethanol. These results indicate that *in vitro* airway tissue models using TEER as a convenient non-destructive endpoint are a promising alternative to animal tests for assessment of subacute 28-day respiratory toxicity and NOAELs. With further *in vivo* correlation and validation, this test may be a useful non-animal alternative for determining safe human subacute exposure levels for inhaled chemicals.

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## Machine learning based meta-analysis using multiple toxicogenomics datasets does not improve genotoxicity prediction

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Toxicogenomics-based approaches using *in vitro* cell models, developed as alternatives to animal testing, have been shown to be able to predict genotoxicity *in vivo* with accuracies above 85%, thereby outperforming the standard test batteries required by regulatory agencies. Despite their performance these approaches are not widely used yet due to the limited number of compounds used, the imbalance in classes, and/or the type of cell model used. In this study, we aim to overcome these hurdles by performing a meta-analysis on the large amounts of toxicogenomics data sets that have been generated the past decade, thereby further improving *in vivo* genotoxicity prediction. From the diXa Data Warehouse, NCBI GEO, and EBI ArrayExpress we collected gene expression data for human, rat and mouse *in vitro* liver cell models exposed to 156, 88 and 44 compounds, respectively, with known genotoxicity information at different time points and dosages resulting in 853, 702 and 100 experiments, respectively. The obtained datasets were merged and pre-processed per species. Species-specific prediction models were built by using 10 machine learning algorithms on 10 train/test sets, each containing 80% of the data for training and 20% for testing. To avoid bias and possibly overfitting experiments with the same compound



were all placed in either the training or test set. Support Vector Machines algorithm had the best accuracy for predicting genotoxicity *in vivo* at 69-82% with 81-93% specificity and 46-61% sensitivity. In conclusion, the meta-analysis did not improve *in vivo* genotoxicity prediction. The saying “the more the merrier, the fewer the better fare” may actually be true here.

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## Transcriptomic alterations in human iPSC derived podocytes exposed to doxorubicin, pamidronate and cyclosporine A

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Glomerular visceral epithelial cells aka podocytes are essential for the function and maintenance of the glomerular filtration barrier. Podocytes play a central role in glomerular disease initiation and progression and their health can be adversely affected in specific genetic diseases, in diseases states such as diabetes and by injury from xenobiotics. Podocytes are notoriously difficult to culture, due to the fact that terminally differentiated podocytes no longer proliferate. Human induced pluripotent stem cells (iPSC), with their capacity for self-renewal offer a potential to provide a continuous source of patient-derived podocytes. The main objective of this work was to investigate for transcriptomic alterations of iPSC-derived podocytes following their exposure to a range of xenobiotics including specific nephrotoxins.

iPSCs were differentiated into podocyte-like cells over 10 days as previously described and cells were characterized based on morphology, immunofluorescence staining for podocyte-specific markers, and VEGF production that was assessed by ELISA. Differentiated podocyte-like cells were exposed to cyclosporine A (1.25 to 15  $\mu$ M), pamidronate (10 to 50  $\mu$ M) and doxorubicin (0.05 to 1.25  $\mu$ M). Quantification of approx. 3500 genes was conducted using the TempO-Seq assay (BioSpyder). After differentiation cells no longer proliferated and exhibited a podocyte-specific morphology with the characteristic “foot processes”. In addition, podocyte markers, including synaptopodin, podocin, nephrin and WT-1 and were expressed and cells produced VEGF after 10 days of differentiation. Transcriptomic analysis demonstrated a concentration dependent impact of a range of compounds and also provided mechanistic insights.

We conclude that iPSC derived podocytes represent a promising tool to identify potential glomerular nephrotoxins.

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## Evaluation of the single-cell level immuno-efficacy of recombinant protein for avian influenza subunit vaccines with a novel AAT integration platform

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Avian influenza virus (AIV) has extensively circulated in migratory waterfowl and poultry. The potential of AIV infecting humans might be a significant threat. Therefore, the development of a new vaccine against AIV is urgent. Subunit vaccines is a new type of vaccine technology. The AIV subunit vaccine do not contain whole virus particles or live components of the influenza virus. It differs from inactivated whole virus vaccines of influenza from chicken embryo eggs, it contains only the antigenic parts of the major surface structure proteins. Even it is only a sequence that would be recognized by a neutralizing antibody (He et al., 2014). Just for this reason, it is advantageous in business and production. Global efforts to promote development of next-generation influenza subunit vaccine, universal and long-lasting protective vaccine. However, the development of next-generation vaccines often requires many experimental animals, and the promotion of alternatives to animal testing (AAT) has become a global goal in recent years. The author's past research, including “Pathogenesis and molecular modeling of avian influenza virus (He et al., 2013)”, “A novel secretory bi-cistronic baculovirus protein expression platform (Hsieh et al., 2018)”, “Modular microfluidic chip control system (He et al., 2015)” and “Peripheral blood mononuclear cell (PBMC) isolation microfluidic chip” were integrated into an AAT project. We developed a molecular modeling process for structural regions of key protein for avian



influenza subunit vaccines. In addition, we prepared influenza virus segmented expression proteins with the baculovirus protein expression platform based on the prediction of molecular modeling. Finally, we tried to use the PBMC microfluidic chip to screen immunodominant, universal and long-lasting protective epitopes of Taiwan H6N1 avian influenza virus. These efforts accelerated the AAT of the avian influenza subunit vaccine development.

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## Human iPSC-derived proximal tubular cells-based transcriptomics data to evaluate cadmium exposure at a high temporal resolution

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Renal proximal tubular cells are one the most susceptible segments of the nephrons to many xenobiotics, including cadmium. In this study, we have explored the utilization of human induced Pluripotent Stem Cells (iPSCs) derived renal proximal tubule-like cells (PTL) as an alternative to other human *in vitro* cell

models to a) investigate their robustness for chemical testing and b) analyze the temporal alterations caused in the pathways affected upon exposure to cadmium.

iPSC derived PTLs were treated with 5  $\mu$ M cadmium chloride (a non-cytotoxic concentration) for 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 16 h, 20 h, 24 h, 72 h and 168 h.

The results showed the early activation of metal response pathway at 1 h which maintained a stable expression level throughout the exposure period of maximum 168 h. Oxidative stress pathway was also activated early at approximately 4 h but started to decrease to baseline levels by 24 h. This demonstrated that iPSC derived PTLs were able to capture the relevant temporal alterations of these two pathways and suggests that they may represent an alternative human-relevant model for *in vitro* toxicity testing.

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## Scoring 3Rs activities of European Member States

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Two official sources of information are currently available to supposedly know what European Member States (MS) are doing to pursue the implementation of the 3Rs at the national level. First source is Article 47 of directive 2010/63/EU published on DG Environment website. Second source is question 4 sent by European Commission (COM) to MS to provide comments and contextualize their annual statistics submitted. Results of question 4 were published along with 2020 statistics report. Based on Article 47, only 15 MS out of 27 have published at least one time their effort for further 3Rs. Based on question 4, a set of criteria developed by the author was used to score progress in 3Rs at MS level. Four aspects were considered: 1) Education and training, 2) Communication and dissemination 3) Funding and awards and 4) National or local 3Rs center. With "1" point allocated per criteria; maximum score was "4". Looking at the overall 3Rs activities i) 56% are actively communicating, disseminating 3Rs principles for end-users ii) 44% are coordinating education and training iii) 24% secure funding and awards for 3Rs iv) 24% have established a national or regional 3Rs centers.

The author will discuss relevance and completeness of the information provided by MS. Moreover, the author will provide an overview of precedent attempts of identifying MS efforts (Taylor, 2014) as well as discuss the development of criteria to go beyond the reductionist analysis based solely on the trends in ani-



mal uses. Last, a way forward will be discussed for national competent authorities and policy makers to take advantage of this scoring system. Ideally, defining criteria can identify good practices and being shared among other MS so that new and specific steps towards replacement are ongoing.

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## Difficult paradigm shift? Reasons for continued animal use for educational purposes revealed in non-technical summaries

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Animals have been considered instrumental in serving as a tool to learn knowledge and skills of living organism functioning as well as in human and veterinary medicine for millennia (Hart et al., 2008). However, animal use in education has been recently criticized by students, scientists, educators, philosophers and policy makers on ethical, economic and environmental grounds (Oakley, 2013; Sapontzis, 1995; Tolbert, 2019). Replacement of animals in education should be easy considering the variety of the non-harmful alternatives that are currently being developed and successfully implemented. And yet, every year thousands of animals continue being used for the purposes of education and training in the European Union alone (European Commission, 2020). The aim of this study was to understand why this is the case. In order to answer this question, we analyzed recently published non-technical summaries, which all EU Member States are obliged to publish in line with the Directive 63/2010 EU. Data from 249 non-technical summaries from 18 EU and EEA Member States published in 2017-2019 revealed that the most often cited barriers to implementation of animal-free alternatives are: 1) practice on living animals is necessary for proper learning or 2) there is no adequate model currently available. In majority of the cases, the latter argument is however invalid, and I will provide specific examples in my presentation to demonstrate that. In conclusion, it is necessary to put a stronger emphasis on engagement with ethical questions that underlie the use of animals and careful consideration of how the learning objectives could be achieved through non-harmful alternatives.

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## The ethics of non-human primate research

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The use of non-human primates (NHPs) for research poses important ethical questions. In recent years, these ethical considerations have led to increased scrutiny and diminished public support of NHP research. Yet, reliance on NHP research continues and, in some scientific domains, is increasing. Ethical considerations are crucial in shaping public policy and in the United States, there is increasing pressure to better align NHP research policy with our current understanding of the ethical implications of this work.

To support this effort, our report "The Ethics of Non-human Primate Research" examines the various ethical features of NHP research from the perspective of different ethical frameworks that influence our moral understanding and, subsequently, public policy. The report opens with an overview of how key ethical frameworks consider issues of harm and moral status, and then examines the ethically salient features of NHP research. By guiding readers through examples of how different ethical frameworks might reflect on the specific features of various research contexts, it provides an opportunity for those outside the field of professional ethics to consider the unique ethical features of NHP research. The ethical analyses provided are grounded in our current understanding of the cognitive and behavioral capacities

of NHPs and how these capacities relate to issues of welfare and well-being.

Careful examination of these ethical considerations suggests that NHP research policy in the U.S. fails to adequately account for important ethical features and that closer attention to concerns about harm and suffering is warranted.

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## ***In vitro* skin irritation protocol for medical devices extracts using EpiDerm model**

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Safety evaluation of medical devices is a complex process and evaluation of skin irritation potential is indispensable part of this process. The rabbit skin irritation test developed by Draize has been successfully replaced by reconstructed human epidermis protocol (RhE) (OECD TG 439). However, this protocol is optimized for neat chemicals and medical device (MD) extracts are dilute solutions with low irritation potential.

To reflect the requirements of ISO 10993 directive, optimized protocol using known irritants and spiked polymers was developed in 2013 (Casas et al., 2013). After successful transfer and standardization, validation scheme was prepared. All 17 laboratories trained in the protocol worldwide produced results with almost 100% agreement with predictions for selected references (Kandarova et al., 2018).

In follow up approach, several medical devices benchmark materials (5 irritants and 2 vehicles) were evaluated in the controlled human patch testing (4 h and 18 h) and in EpiDerm *in vitro* skin irritation protocol. Results were then compared to existing rabbit skin irritation test data.

Based on the preliminary studies an international round robin validation study was conducted in 2016 to confirm the ability of the RhE models to correctly predict the intra-cutaneous irritation of extracts from medical devices (4 irritants and 3 non-irritant materials). Blinded polymer samples were extracted with sesame oil and saline according to ISO 10993-12 (De Jong et al., 2018).

The protocol employing EpiDerm tissues was able to correctly predict virtually all of the irritant polymers in the saline, sesame oil as well as in both solvent extracts. Our results confirmed the ability of *in vitro* approach using RhE tissue models to detect the presence of skin irritants at low concentrations in dilute medical device polymer extracts (De Jong et al., 2018). The use of the reconstructed tissue models, as replacements for the rabbit

intra-cutaneous test is implemented into the ISO 10993-23:2021 standards used to evaluate medical device biocompatibility.

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## ***In vitro* cell transformation biomarkers as criteria for evaluating potential carcinogens**

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Assays for neoplastic cell transformation (CTA) imitate some of the *in vivo* steps of multi-stage carcinogenesis process and can detect both genotoxic and non-genotoxic carcinogens *in vitro*. But there are a number of issues that hamper consensus on test approval. The proposed assessment of transformed cells in culture in the described methods is purely subjective in nature (morphological features, cell orientation relative to each other). Thus, the development of new approaches to the identification of transformed cells will increase the prognostic efficiency of CTA.

The aim of this work was to investigate the possibility of using biomarkers of cell transformation in culture as criteria for evaluating potential carcinogens in a test for neoplastic cell transformation.

Cell lines of transformed and normal embryonic muscle-skin fibroblasts of mice, a Syrian hamster were obtained and characterized. Cultures of transformed cells were obtained at passage 8-12 of culturing cells from the “foci” of the cell layer with signs of transformation (random orientation of cells in a monolayer, lack of contact inhibition between cells and the formation of “foci” of cells).

A comparative analysis of the levels of proliferative activity was carried out. Histochemical indices for cadherin and integrin were established. The analysis of changes in the total area of the colonies and the morphology of transformed and normal cells after 8 days of cultivation was carried out.



The data obtained led to the conclusion that a change in indicators such as proliferative activity (an increase in the mitotic index by an average of 17%), an increase in the number of cells and the area of colonies, the level of ROS in the cells, adhesive ability (changes in the expression of adhesion molecules) are characteristic features of transformed cells and can be used as additional markers and criteria in CTA.

**Presentation:** Posters

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### Trichloroethylene glutathione conjugates: Biotransformation kinetics and impact on oxygen consumption rates (OCR) in human renal proximal tubular cells (RPTEC/TERT1)

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Trichloroethylene (TCE) is a high-production volume chemical that has been widely used for decades and poses a significant hazard to human health (Cichocki et al., 2016). Animal and *in vitro* studies have demonstrated that hepatic glutathione conjugation of TCE by glutathione S-transferases (GST) and subsequent renal metabolism involving  $\gamma$ -glutamyl-transferase (GGT), cysteinylglycine and cysteine conjugate  $\beta$ -lyase activity can lead to the formation of a reactive thioetene, causing oxidative stress and mitochondrial injury (Birner et al., 1997; Cummings and Lash, 2000; Lash et al., 2014).

Here, we re-examined the hepatic GSH-conjugation of TCE and its effects on human renal proximal tubule cells. By using human liver fractions and recombinant human GSTs we demonstrate the formation of trans 1,2-dichlorovinyl-glutathione (trans 1,2 DCVG) and 2,2-dichlorovinyl-glutathione (2,2 DCVG). RPTEC/TERT1, (Wieser et al., 2008) were differentiated and treated with trans 1,2 DCVG and 2,2 DCVG. A fast and complete metabolism of the initial GSH-conjugates was observed within 2 hours, with a temporal transient increase of the respective cysteine-glycine conjugate (DCV-cys-gly), followed by the formation of the cysteine conjugate (DCVC). LC-MS-TOF was used to monitor the biotransformation of the GSH-conjugates over 24 h. The specific effects of the two GSH-regioisomer conjugates on mitochondrial respiration were quantified by using a Seahorse bioanalyzer. A decreased in oxygen consumption rate (OCR) and redox capacity was observed for trans 1,2 DCVG, but not in the presence of  $\beta$ -lyase inhibitor aminooxyacetic acid (AOAA). On the other hand, 2,2 DCVG showed no effect for the same conditions.

This study provides new insight regarding the molecular processes of TCE metabolism and toxicity in human renal cells

demonstrating a clear relationship between metabolism and isomer-conjugate dependent mitochondrial perturbation.

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**Presentation:** Posters

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### Liver spheroid co-cultures with fresh or cryopreserved hepatocytes and endothelial cells as tool to investigate metabolism and hepatotoxicity

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Primary human and animal liver cells are the gold standard for all pharmacological-toxicological *in vitro* studies during drug development. Three-dimensional (3D) cultures became more popular in recent years since they might mimic the *in vivo* cell morphology, polarity and cell-cell interactions better than traditional two-dimensional (2D) cultures. Due to better access and continuous availability, cryopreserved cells become more popular, but functional differences to fresh hepatocytes might occur.

Here, 3D spheroids were generated in U-bottom ULA (ultra-low attachment) plates with fresh and cryopreserved human hepatocytes as single culture, and in co-culture with liver endothelial cells to display an even more physiological situation. The metabolic activity of different Cytochrome P450 activities and the



acute toxicity for known substances like acetaminophen were tested and compared for fresh and cryopreserved cells. Spheroids from fresh and cryopreserved human hepatocytes were inducible for CYP activity, but a strong variability of basal CYP activity and CYP-induction potential was detected between different donors especially in fresh hepatocytes. A higher CYP activity and CYP inducibility was determined in 3D-cultures compared to 2D. In total, CYP inductions demonstrated a better and more stable inducibility in spheroids from cryopreserved cells. The ATP assay displayed almost the same concentration dependent toxicity for acetaminophen in 3D and 2D cultures. In contrast, the AlamarBlue assay didn't show this dependency for spheroids, here only the highest concentration led to a decrease in viability.

Our results indicate that differences may exist between 3D-cultures with fresh and cryopreserved hepatocytes and in comparison to standard 2D-culture models. These differences may lead to different and conflicting results in the assessment of drug toxicity and drug-drug interaction.

**Presentation:** Posters

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## A novel polymer generates cell repellent surfaces and allows 3D cell culture

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Once isolated, primary cells are usually placed in an artificial environment in protein coated plastic or glass containers, where they assemble as 2D layer, reshaping their morphology and behavior. Limitations in such 2D cultures have been increasingly recognized and different systems have been established which allow growing primary cell as 3D spheroid. Low attached surfaces are chemical defined and can be easily handled, but remaining interaction of cell and surface coating may impact the morphology and behavior of the cell spheroid. Here, a cell repellent surface with Biofloat solution was generated in uncoated non-tissue culture treated U-bottom plates. The plates were used to assemble 3D spheroids with cryopreserved non-human primate hepatocytes. These cultures were compared to spheroid cultures in U-bottom ULA plates available on the market. Time for spheroid formation and functionality of 3D cultures in terms of metabolic CYP P450 activities and their inducibility were investigated. All products worked to grow spheroids. Round spheroids with clear borders were formed. Clear differences could be found in shape and kinetic of spheroid formation for the different products. Pre-coated benchmarks tend to form spheroids, which were less regular and needed more time to form a compact aggregate. This is likely due to remaining adhesion points and interaction

with the plastic surface on plates which are not fully inert. One should carefully choose the equipment as this has a major impact on quality of the cell aggregates. Spheroids from cryopreserved Cynomolgus hepatocytes were inducible for CYP activity in a similar extent in Biofloat plate and all other tested ULA plates. These results indicate that Biofloat is an optimal polymer formulation to generate cell repellent surfaces and thereby making 3D cultures possible. The coating provides the possibility to attach functional groups anchored in a fully inert background which are usable for other cell culture purposes.

**Presentation:** Posters

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## Hepatic non-parenchymal cells isolated from rodent livers and characterized for the development of primary hepatic co-cultures

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Liver cells are established as *in vitro* models for toxicology studies in 2D and 3D cultures. The goal is to approach the *in vivo* situation as good as possible. To expand the specificity of the liver models, the total cell population of the liver has to be taken into account. Cultivation and verification of these non-parenchymal cells, like Liver Sinusoidal Endothelial Cells (LSEC), Kupffer cells (KC) and Stellate cells (SC) remain an issue. Percoll gradients for cell separation of non-parenchymal cells have been established, but the yield of NPC per gram liver tissue can be low. At PRIMACYT, internal experiments comparing Percoll- and Iodixanol gradients in rat liver showed a slightly higher yield (20%) of the whole NPC population when cells were isolated by an Iodixanol gradient compared to Percoll. In mice, this effect was more obvious by providing about 54% higher NPC yield with Iodixanol. We examined assays to prove purity and viability of the KC, LSEC and SC fractions of the rats and mice after purification by Iodixanol. In a first step, Kupffer cells were tested over culture time for their stimulation by LPS, followed by a phagocytosis test. LSEC fractions were tested equally to proof of the absence of phagocytic cells. Stellate cells were tested for the presence of lipid droplets by Oil Red O staining. Further analysis was done by immunofluorescence by CD68 (KC), CD31 (LSEC) and Vimentin (SC). Finally, cells were tested for RNA markers CLEC4F (KC), TIE1 (LSEC) and POSTN (SC) to get a clear indication of the purity of the cell fractions. Together, the tests could give rise to a clear characterization of the liver cells. Finally, the approach to utilize tissue-like co-cultures should reduce the number of laboratory animals used for toxicity studies and drug development.

**Presentation:** Posters



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## Impact of culture conditions on the expression and function of bile acid transporters in primary rat hepatocytes

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In primary hepatocytes, the expression of membrane transporters like the bile acid transporters NTCP (Slc10a1) and BSEP (Abcb11) is rapidly downregulated in culture. Therefore, experiments concerning transporter function should be carried out as soon as possible after isolation. We analyzed the impact of different culture conditions on the expression and function of both transporters in primary rat hepatocytes. The cell suspension was directly plated after isolation or kept on ice for 24 h in cell preservation solution before plating the next day. Transporter expression was analyzed 2 and 4 days after isolation using qPCR and western blot, as well as on functional level for NTCP using Cholyl-L-Lysyl-Fluorescein (CLF) as substrate. No significant differences in transporter expression were detected between freshly prepared hepatocytes and cells stored for one day on ice. However, both transporters were dramatically downregulated after two and four days in culture on mRNA and protein level. In contrast to the directly plated cells, hepatocytes seeded after 24 h on ice showed a higher protein level for NCTP and BSEP two days after preparation. These results are in line with our transport studies indicating a 30% enhanced uptake of CLF into cells stored on ice for 1 day compared to directly plated ones on day two after preparation. The active uptake of CLF could be better inhibited by Rifampicin in cells plated on day 1 after isolation. These results suggest that the storage of hepatocytes on ice for 24 h supports and maintains transporter expression. For studying NTCP- and BSEP-mediated transport, this modified protocol was superior to regular culture until day 2. These findings should be taken into account when using hepatocytes from external suppliers: ordering of cells in suspension instead of plated hepatocytes seems to be advantageous when bile acid transporter activities are studied.

**Presentation:** Posters

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## Three-dimensional culture improves the morphology and function of cardiomyocytes derived from human pluripotent stem cells

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So far, we have been conducting animal tests or simple *in vitro* assays for preclinical studies in drug development. However, the animal models often cannot recapitulate an entire disorder or disease, and the human system and *in vitro* assays are too simple to reproduce the detailed physiological features observed *in vivo*. The cells derived from a human pluripotent stem cell (hiPSC) offer important advantages for drug toxicity screening against relevant human organ cells and may facilitate drug development. Especially, the hiPSC-derived cardiomyocyte (hiPSC-CMs) represents a potentially powerful tool to model aspects of heart physiology relevant to disease and adverse drug effects. However, two-dimensional (2D) monolayers of hPSC-CMs are limited in their ability to mimic native cardiac tissue structurally and functionally. To elucidate the physiological changes according to culture conditions, we compared the morphological features with microscopy imaging analysis and electrophysiological functions with cardiac action potential and the relative gene profiling in 2D- or 3D-cultured hiPSC-CMs. In this study, we found that 3D culture induced the intracellular morphological changes of hiPSC-CMs with the increased population of organized myofilaments and highly quantified mitochondria. The 3D culture also enhances the action potential parameters in electrophysiological analysis, which is consistent with the result of the increase in cardiac ion channel expression important to cardiac function. These results demonstrate that the 3D culture improves the phenotypic maturity and electric functionality of hiPSC-CMs.

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**Presentation:** Posters



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## Successful development of recombinant human diphtheria antitoxin: A project update

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Diphtheria antitoxin (DAT) is a life-saving drug, but the way it and other antitoxins are manufactured hasn't changed in more than 100 years. As with most therapeutic antitoxins, DAT is produced from the serum of equines who have been hyperimmunized by repeated toxin injections. In addition to documented animal welfare problems at facilities where hyperimmunized equine serum is produced, equine antitoxins can cause adverse health effects in humans who receive them. In recent years, public health authorities have noted a global shortage of equine DAT and called for the development of alternative products. As a first step toward developing a non-animal replacement for equine DAT, the PETA International Science Consortium Ltd. funded the development of human monoclonal antibodies against diphtheria toxin that can be produced in cell culture. In 2017, we introduced this project and presented early results at Word Congress 10. Here, we report the successful results of the completed project, which has led to the development of a set of fully human diphtheria toxin-neutralizing antibodies that are ready for clinical development (Wenzel et al., 2020). The process used in this project serves as a template for other groups interested in replacing animal-derived therapeutic and diagnostic antibodies with human recombinant antibodies.

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**Presentation:** Posters

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## The importance of simulated lung fluid composition for *in vitro* solubility of mineral wool fibers

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In the European Union, vitreous fibers are classified as a potential carcinogen to humans, thus they have to fulfill certain safety cri-

teria to be placed on the market. Carcinogenicity was proven to be driven by fibers biopersistence in the lungs (Bernstein et al., 2001). Based on the *in vivo* results, fibers can be exonerated from being classified as carcinogenic (EC 1272/2008), if their clearance from the lungs is fast enough, i.e., weighted fiber half-life should be less than 10 or 40 days, for inhalation and intratracheal instillation test, respectively. Thus, it is important that fibers are biosoluble. The solubility of fibers could be assessed by *in vitro* tests and many methods using different simulated lung solutions for acellular *in vitro* testing of inhaled materials have already been developed and proposed (Sebastian et al., 2002; Cannizzaro et al., 2019; Marques, 2017) however, solutions that simulate lung fluids used for the test vary greatly in the published literature.

In our study, the influence of several simulated lung solutions, mimicking the extracellular lung environment, on the dissolution of glass and stone mineral wool fibers were evaluated. The solution formulation varied from simple inorganic to the one containing the most abundant representatives of organic found in extracellular lung fluid. Solution composition was proven to be affecting the solubility of both glass and stone mineral wool fibers. Buffering capacity of the solution and the presence and content of organic compounds were shown to be the crucial parameters.

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## Simulation of dystocia in bovine and equine using models preserved by the Elnady Technique

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Among the major challenges encountered during parturition in bovine and equine is dystocia. Dealing successfully with such cases depends mainly on the veterinarian's level of technical skills. This work aimed to enhance the training of veterinary stu-



dents and novice practitioners in how to deal with various cases of dystocia in large animals. Effective training can minimize the stress on the fetus and the dam. We preserved the cadavers of a full-term foal, donkey, and two calves using the Elnady Technique. The developed models are realistic, relatively flexible, durable, dry, and have no offensive odor. Phantoms for holding the specimens were constructed for the dam. Together, the training models allow the dystocia cases to be presented and corrected. They showed a high fidelity and were well received by both the students and the instructors.

**Presentation:** Posters

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## Differentiation and upregulation of specialized epithelial cells in porcine and human intestinal organoids

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**Background and Objectives:** Due to anatomical and physiological similarities, the pig is a popular model for translational research in gastrointestinal diseases. Beside many similarities, there are species-specific differences in the pathophysiological outcome of diseases like the infection with *Yersinia enterocolitica*, which causes severe diarrhea in humans while pigs remain mostly asymptomatic. In order to study underlying mechanisms, intestinal organoids have become an important model. This study aims to differentiate and overexpress specialized epithelial cells in human and porcine intestinal organoids for investigation of molecular mechanisms in zoonoses.

**Material and Methods:** Human intestinal organoids were differentiated from human-derived induced pluripotent stem cells while porcine intestinal organoids were generated from porcine jejunal crypts. Organoids of both species were first cultured in long-term culture medium containing Wnt3a, R-Spondin and Noggin. In different experiments, following adjustments to the medium were made: IGF-1 and bFGF were added to achieve a more diverse and therefore more physiological epithelial cell population (Fujii et al., 2018). Further experiments aimed to differentiate M-Cells by adding RANKL (Rouch et al., 2016), goblet cells by adding DAPT and enteroendocrine cells by adding DAPT, IWP-2 and PD0325901 (Basak et al., 2017). The organoids are analyzed for the expression of characteristic markers for specialized epithelial cells via RT-qPCR and immunofluorescence staining.

**Results:** RT-qPCR results show an upregulation of cell-specific marker genes in experiments with porcine intestinal organoids. Experiments with human intestinal organoids as well as immunofluorescence staining are ongoing.

**Discussion and Conclusion:** Upregulation of cell-specific marker genes indicates upregulated expression of the epithelial cells aimed for in the differentiation protocols. Further analyses will show how these cells are arranged and whether they are functional so that these organoids can be used to study pathomechanisms.

**Acknowledgement:** The project is funded by the H. Wilhelm Schaumann Stiftung.

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**Presentation:** Posters

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## Cosmetic Europe's long range science strategy non-animal safety assessment case study of phenoxyethanol in cosmetics

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The Cosmetics Europe Long Range Science Strategy (LRSS) is performing several case studies to evaluate the practical application of Next Generation Risk Assessment (NGRA) for cosmetic ingredients. The present work is an exposure led NGRA case study

for the preservative ingredient phenoxyethanol. The case study was guided by the SEURAT-1 safety assessment workflow (Berggren et al., (2017) and the International Cooperation on Cosmetics Regulation (ICCR) NGRA principles (Dent et al., 2018), with the aim of using only non-animal approaches to assure the systemic safety of this ingredient when present at an active level (1%) in a product with a high level of consumer use (body lotion). This strategy is aligned with the US EPA's next generation blueprint for toxicology, which seeks to characterize whether a chemical acts via defined biological pathways/targets or if it may induce cellular changes by a non-specific mechanism (Thomas et al., 2019). PBK modelling was performed to estimate the internal (plasma) concentration of phenoxyethanol in consumers following use of the product. Population modelling, taking into account known genetic polymorphisms, provided a 95<sup>th</sup> percentile population C<sub>max</sub> of 6.3 µM for phenoxyethanol. *In vitro* bioactivity data including high throughput transcriptomics, the ability to interact with specific molecular targets and data evaluating cellular stress were generated and Points of Departure (PoDs) were compared with the internal exposure estimations. The relevant PoD for risk assessment was the lowest pathway No Observed Transcriptional Effect Level (NOTEL) BMDL10 in HepG2 cells of 171 µM. Sources of uncertainty were identified and characterised to enable the level of confidence in the safety assessment to be assessed. The PoD identified was 27 times lower than the 95<sup>th</sup> percentile C<sub>max</sub> of phenoxyethanol.

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**Presentation:** Posters

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## The RTGILL-W1 cell line assay to predict fish acute toxicity of water samples and chemicals (ISO 21115:2019)

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The acute fish toxicity test is one of the most commonly performed vertebrate tests in the environmental risk assessment of chemicals and water samples. Fish are exposed to different concentrations and deaths within a 96hour period are recorded as the endpoint. Annually, millions of fish are sacrificed for this test, which, together with its large resource need motivates the need for alternative testing procedures. Assuming that the gills of fish are the major target organ during short term exposure, we have developed an *in vitro* assay based on the rainbow trout (*Oncorhynchus mykiss*) cell line, RTgill-W1. In this assay, cells are exposed to chemicals in 24-well plates for 24hours in a fully defined exposure medium, L-15/ex. Exposure concentrations are verified at the onset and end of exposure, and effect data (cell viability) are calculated based on measured chemical concentrations. Cell viability is assessed by means of three fluorescent indicator dyes. These are applied to the same set of cells, targeting metabolic activity, cell- and lysosomal- membrane integrity. Two independent validation studies (Natsch et al., 2018; Tanneberger et al., 2013) with over 70 organic chemicals of varying physico-chemical properties, modes of action and toxicities demonstrated the high predictive capability of this test to forecast acute fish toxicity (i.e., LC50 values). Numerous municipal and industrial water samples have been explored as well. Repeatability (intra-laboratory variability) and reproducibility (inter-laboratory variability) of the assay have been demonstrated in a round-robin study focusing on six test chemicals and involving six laboratories from the industrial and academic sector (Fischer et al., 2019). As the obtained results confirmed the accurate and robust performance of this test, the International Standardization Organization (ISO) has recently approved the assay as the first international standard based on a fish cell line (ISO 21115:2019) – rendering it a fore-runner in this regard.

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ISO 21115:2019 Water quality – Determination of acute toxicity of water samples and chemicals to a fish gill cell line (RTgill-W1). International Standardization Organization.

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**Presentation:** Posters

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## E-learning modules to promote implementation and development of new alternative approaches to animal testing: Content development and potential integration into training courses

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Since the publication of the Directive on the Protection of Animals used for Scientific Purposes (EU Directive/2010/63), the European Commission has supported several initiatives to harmonize its interpretation and implementation. One such initiative was the creation of online learning modules to (1) facilitate the identification of existing non-animal approaches and (2) assist in the development of new alternative approaches among academic laboratories, test system suppliers, industry and educational bodies. Content providers with experience in systematic searches, developing and validating new approach methodologies, and e-learning content creation have collaborated to develop these modules for presentation on the Education & Training Platform for Laboratory Animal Science (ETPLAS). Content of the modules, including learning outcomes, and demonstrations of how the project has translated regulatory guidance into accessible training materials to harmonize interpretation and aid in the speed of implementation will be presented. Suggestions for, and examples of, potential integration of these modules into academic courses and job specific industry trainings are provided.

**Presentation:** Posters

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## hPlacentox: New method for endocrine disruptor assessment using a human placental model

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**Background and Objectives:** Endocrine disrupting chemicals constitute an important public health issue, since more and more substances are suspected to be endocrine disruptors. Endocrine disruptors (ED) are defined by the World Health Organization (WHO) as exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations. Most of existing tests dedicated to the assessment of endocrine disruption only focus on hormonal disturbance and none of them evaluates both hormonal disturbance and adverse effects. Our objective was to develop a human placental cell model for the evaluation of endocrine disruption potential according to WHO definition. Placenta supports the normal growth and development of the fetus by coordinating exchanges of nutrients and wastes between maternal and fetal circulatory systems and by secreting numerous hormones. Placental dysfunctions are therefore commonly associated to pregnancy disorders. Amongst pregnancy disorders, preterm birth has been associated to the purinoreceptor P2X7.

**Materials and Methods:** We previously selected the human placental JEG-3 cell line and optimized culture conditions to high-light chemicals cytotoxicity and named our cell model JEG-Tox (Olivier et al., 2021). The release of polypeptide hormones hPL and hCG was quantified by ELISA and steroid hormones estradiol and progesterone release by fluorescence energy transfer. The activation of P2X7 receptor was measured by the YO-PRO-1 assay.

**Results:** Bisphenol A, dibutylphthalate and 3-benzylidene camphor that are all established endocrine disruptors induced P2X7 receptor activation and increased or decreased hormones release.

**Discussion and Conclusion:** Endocrine disruptors (bisphenol A, dibutylphthalate and 3-benzylidene camphor) triggered the activation of P2X7 degenerative receptor and altered hormones release in JEG-Tox cells. Based on those results, our method hPlacentox has been selected by the European academic-private PEP-PER platform to enter the OECD pre-validation process for the assessment of endocrine disruptions.

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**Presentation:** Posters



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## Towards an ADME competent 4-organ-chip

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Complex human *in vitro* ADME models involving co-culture of key organs to mimic certain exposure routes present a challenge to establish physiologically relevant organ models as well as physiologically based pharmacokinetic (PBPK) distribution behavior in the culture environment. In our recent study, we developed a PBPK compliant ADME 4-Organ-Chip (Chip4) with a downscale factor of 1:100,000 of human body. The integration of an intestinal barrier model for absorption, liver microtissues for the main metabolism, a kidney model with proximal tubular-like cells and podocytes for excretion, and neuronal spheroids as a potential target organ were optimized in the chip and co-cultured for 14 days. We exposed the Chip4 to Haloperidol, an antipsychotic medication in butyrophenone family and to Carbamazepine, a tricyclic compound with anticonvulsant properties through different routes with a repeated dose to observe their metabolite induced toxic effects on an organ-specific level. We aim to develop a testing system as a potential new approach methodology for toxicological testing and to increase predictability in the preclinical stage with the multi-organ-chip platform.

This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No: 681002.

**Presentation:** Posters

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## A novel epidermis equivalent for *in vitro* skin irritation testing: Characterization and performance

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Rebuilding epidermis has been of interest to replace animal models for toxicological studies, and six reconstructed human

epidermis (RHE) were successfully validated to investigate *in vitro* potential skin irritants (OECD Test Guideline N°439). However, the use of these RHE models has a high degree of dependability in the commercial strategies of the suppliers, and they have limited accessibility for companies of countries with customs barriers. Thus, this study reports the development and characterization of a novel epidermal equivalent meant to be used for *in vitro* skin irritant testing. For developing the in-house RHE, we have modified published protocols (Poumay et al., 2004; Pedrosa et al., 2017). The in-house RHE, constructed with neonatal KCs (nKCs) plated on collagen IV-coated inserts, presented a well-differentiated epidermis (~ six layers of differentiated viable cells, mature stratum corneum, 64.5 µm thickness), and resembles human epidermis (Hematoxylin staining). This model also demonstrated similarities to native human epidermis in terms of marker expression (Keratin-10, Keratin-14, Filaggrin, Involucrin). Parameters of cell viability (optical density average at 570 nm of 1.6) and barrier function integrity (IC50 of 3.23 mg/mL – sodium dodecyl sulfate) match the quality control criteria adopted by OECD 439. The performance of in-house RHE as skin irritation model was evaluated by the SkinEthic™ test method with 19 reference substances (OECD 439); and results shown that the in-house RHE was able to discriminate between irritating and non-irritating substances. Moreover, its performances as a skin irritation test were similar to the ones described in the OECD 439 (Alépée et al., 2010; Kojima et al., 2013; OECD 439). Taken together, these results demonstrate the potential use of a novel RHE (in-house RHE) as skin irritation model especially for those countries in which validated RHEs have limited accessibility.

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**Presentation:** Posters



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## An innovative millifluidic 3D human trabecular cell *in vitro* platform as tool to mimic the pressure variations observed in open angle glaucoma

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Glaucoma is a chronic, progressive and heterogeneous optic neuropathy marked by progressive optic nerve atrophy and retinal ganglion cell death. Up to today, the most common glaucoma risk factor is elevated intraocular pressure (IOP) and it is also the only clinically modifiable one in glaucoma treatment. However, this approach often fails because it does not offer a complete protection towards the sight deterioration (Overby et al., 2009; Yang et al., 2018).

Therefore, a better understanding of both the generation and the regulation of the outflow resistance could improve the knowledge of glaucoma therapies.

A significant body of evidence suggests that the inner wall endothelium of Schlemm's Canal and the juxtacanalicular connective tissue, which is a part of trabecular meshwork (TM), are responsible for the intraocular pressure homeostasis maintenance (Stamer and Clark, 2017). However, in glaucomatous eyes, due to TM damage (i.e., changes in the extracellular matrix deposition), there is a lack of this proper control and an "extra" outflow resistance is generated.

To set up an innovative *in vitro* model to investigate the early molecular mechanisms triggered by pressure increase on a 3D model of human TM cells (HTMC), we fine-tuned our platform of milli-scaled multi-organ device in a single flow configuration (Saccà, 2020) by adding an auxiliary device to simulate the circadian increased pressure flow (IPF). The HTMCs were subjected daily to a 10% IPF for 10 h/day, up to 7 days.

Preliminary data evidenced that IFP boost acts as mechanical stress on TM, inducing an up-regulation of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\alpha$ ), profibrotic markers (SPARC, TGF $\beta$ 2) and MMPs gene levels, all strictly linked to ECM turnover. Taking into account these preliminary findings, our innovative *in vitro* devised model could provide a useful tool to discover new molecular targets to restore outflow resistance and/or the TM functions.

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## Presentation: Posters

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## 3Rs principles in animal ethics and experimentation: How a methodology to analyze micronucleus can influence the excellence of laboratory and reduce animal numbers

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The 3R's Principle was proposed by William Russell and Rex Burch in 1959, requesting the replacement, reduction, and refinement of experimental methods. Therefore, in the last two decades the development of alternative methods to animal experimentation has been progressing rapidly. *In vitro* methods are extremely important in the evaluation of toxicological substances, especially those already validated by regulatory agencies (Kandárova e Letasiová, 2011). Among the various methods, *in vitro* mutagenicity tests were the first accepted for regulatory purposes (Liebsch and Spielmann, 2002). Following the global trend and together with the publication of the new regulatory framework in Brazil (RDC 294 in July 2019), the Con-

tract Research Organization (CRO) had to adapt. In this sense, our laboratory implemented the *in vitro* micronucleus analysis, then replacing the previous method, *in vivo*. During the first year, the analyses were performed by conventional microscopy, whose disadvantage was their prolonged time to analysis and the requirement for specialists, resulting in customer dissatisfaction and personnel costs. On the other hand, this substitution has saved about 7000 animals previously used for *in vivo* analysis. After its automation (flow cytometry), it was possible to reduce the experimental period to 60 days, and increase the laboratory production capacity, previously from 3 to 4 studies per month to 16 to 20. Therefore, the implementation of such methodology and its automation led not only to the reduction in the number of animals used, as well as fast and accurate results promoting increased visibility in the market and satisfaction of our customers.

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**Presentation:** Posters

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## Proof-of-principle for impedance measurement with carbon electrodes for implementation in a bone-on-a-chip system

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Organ-on-chip (OoC) systems are a promising alternative to the still common animal experimentation. However, when compared to OoC systems found in literature such as lung, liver or kidney, bone is underrepresented as indicated by the rather low number of proposed systems (Scheinpflug et al., 2018). One reason is that the development of bones *in-vitro* is a time-consuming process that can take up to several weeks. Current-

ly, the process of matrix mineralization can only be investigated by end-point assays that require the destruction of the organoid. Therefore, a sensor-based, reliable and robust method for a non-invasive and continuous determination of *in vitro* matrix mineralization would be of great advantage, especially when 3D constructs are cultured in microphysiological bioreactors. In the presented work, we show data from impedance measurement on a collagen scaffold-dummy with osteogenic medium (Schulze et al., 2014). Measurements were performed in a bioreactor (ID = 13 mm, height = 3mm) which was sandwiched by two impedance foils (Bio24, well B3 with passivated feed line). For impedance measurement a VersaSTAT 3 was used. Impedance spectroscopy from 1 Hz to 1MHz with an RMS of 30 mV was performed. The setup was mounted in a Faradic cage which was heated to 37°C. With the performed measurement we were able to distinguish pure osteogenic medium from a medium soaked collagen scaffold by approximately 60  $\Omega$  in the real part. During the performed proof-of-principle measurement no clear difference in the imaginary part of the impedance was visible. Further measurements with mineralized bone tissue are planned to further investigate the possibilities of the technology. The results indicate that the proposed geometry can be used to perform impedance measurement at bone scaffolds. Furthermore, a fixed frequency of 10kHz and 30mV seems to be suitable for the measurement (Schmidt et al., 2020).

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**Presentation:** Posters



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## New insights on alternatives in medicinal mushroom research: *In silico* predictive evaluation of epigenetic modulatory events and anti-inflammatory potential of polyphenols

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*In silico* models emerge as a popular non-animal method to predict biological activities, due in part, to worldwide pressure to reduce, refine and replace the use of animals in science.

Mycotherapy is a promissory source of agents with immunomodulating, anti-inflammatory and antitumor properties (Morris et al., 2017). We highlight the utility of *in silico* predictive evaluation of epigenetic modulatory events and anti-inflammatory potential of mushrooms polyphenols by an approach consisting of molecular docking coupled to drug-likeness and ADME/T study (absorption, distribution, metabolism, excretion and toxicity).

The molecular docking between phenolics and six enzymes – two DNA methyltransferases, two histone acetyltransferases and two histone deacetylases – was carried out. Free energy ( $\Delta G$ ), dissociation constant ( $K_i$ ) and ligand efficiency (LE) were estimated. In the docking with DNA methyltransferases hesperetin showed the strongest interaction in terms of  $\Delta G$  -10.01 Kcal/mol,  $K_i$  45.6 nM and LE -0.46. For histone acetyltransferases, the myricetin model showed the robust interaction,  $\Delta G$  -9.60 Kcal/mol,  $K_i$  92.3 nM and LE of 0.48. All models achieved similar results for histone deacetylases. The molecular interactions between these enzymes with their phenolic ligands, suggested their potential modulating effect on relevant epigenetic events in cancer chemoprevention.

The interactions between polyphenols and four proteins involved in inflammation: the transcriptional factor NF- $\kappa$ B, and the enzymes NADPH oxidase, 2-cyclooxygenase (COX-2) and 5-lipoxygenase (5-LOX) was also studied. The results showed the potential of the quercetin and catechin as inhibitors of NF- $\kappa$ B, NADPH oxidase, and COX-2 and, particularly, of homogentisic acid as inhibitor of 5-LOX. The homogentisic acid displayed the best results in terms of drug likeness and ADME/T tests.

We demonstrate the utility of a computerized workflow to identify the structural basis for the interaction of polyphenols

with molecular targets of epigenetic signaling and inflammation. Thus, alternatives will offer perspectives when addressing efforts for ethics and lab animals' welfare.

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**Presentation:** Poster

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## Quantitative sensitizing potency assessment using GARDskin dose-response

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Proactive identification and characterization of sensitization hazards are central aspects of risk assessment of chemicals. Current legislations and trends in predictive toxicology advocate a transition from *in vivo* methods to non-animal alternatives, with a number of methods for hazard assessment of skin sensitizers currently available. However, non-animal methods capable of providing quantitative assessment of sensitizing potency are currently lacking.

The GARDskin assay is a next-generation *in vitro* assay for hazard assessment of skin sensitizers, currently progressing towards regulatory acceptance. Recently, the GARDskin Dose-Response (DR) testing strategy was introduced, in which test chemicals are evaluated by the GARDskin assay in a titrated range of concentrations, in order to investigate the dose-response relationship between GARDskin classifications and test chemical concentration. As such, it provides a quantitative estimation of sensitizing potency, referred to as cDV0, which corresponds to the least required dose able to generate a positive response in the GARDskin assay. The cDV0 value obtained for a test chemical may be viewed as an analogue to the LLNA EC3 value, based on which further hazard characterization and risk assessment may be performed. Statistically significant correlation between the GARDskin DR cDV0 and the LLNA EC3, as well as with human No Expected Sensitization Induction level (NESIL) estimations has been confirmed, thus enabling direct extrapolation between the different metrics.

Here, we further illustrate how these results can be used on their own to facilitate direct potency-associated ranking of test chemicals. Furthermore, we demonstrate how obtained cDV0 values can be extrapolated to LLNA EC3 values with a 95%



confidence interval, thereby also facilitating potency-associated subcategorization of test chemicals according to UN GHS classification criteria. Lastly, we illustrate how results generated with GARDskin DR can be directly incorporated into existing strategies for Quantitative Risk Assessment using an entirely *in vitro* setup.

#### Reference

Gradin, R. et al. (submitted). Quantitative sensitizing potency assessment using GARDskin dose-response.

**Presentation:** Poster

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## Educators' views on dissection alternatives at the onset of the COVID-19 pandemic

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When the COVID-19 pandemic struck in 2020, many schools made the decision to cancel face-to-face classes and move instruction online. To better understand how the pandemic affected science educators' plans to conduct classroom animal dissection exercises, we conducted a nationwide survey of biology teachers ( $n = 2,131$ ) and asked about their experience as classes transitioned online. Our survey revealed that 72% of biology educators had planned on having their students participate in classroom animal dissection exercises in the spring of 2020. Of those educators, 29% shifted to the use of dissection alternatives, such as web-based programs, as a result of remote learning. Our survey investigated which alternatives were most used, whether teachers were already familiar with the alternatives, how teachers identified those alternatives, and whether the educators planned to use dissection alternatives again for in-person or online learning. These survey results provide insight into biology educators' use of dissection alternatives during the COVID-19 pandemic as well as their post-pandemic plans and may increase awareness and usage of dissection alternatives within the educational community.

**Presentation:** Poster

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## Development and characterization of the Skin Surface Temperature Modulation and Control (Skin-Temp-MC) device

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The relationship between skin function and barrier properties depends among others on skin surface temperature (SST) and environmental conditions. Temperature regulation and water transport are both important functions. Changes in SST are known to depend on, e.g., age, gender, body region, blood flow, agitation, day-time and season of the year (Yosipovitch et al., 1998; Batinga et al., 2015). In summary, the *in vivo* temperature fluctuates around the physiological relevant temperature of 32°C, which is reflected in the 3R method on *in vitro* skin absorption set to 32°C ± 1°C. Accordingly, a fast and accurate monitoring and controlling the SST is demanded to evaluate temperature effects and interactions with topical formulations.

The Skin-Temp-MC device is equipped with a contact-less IR or miniature resistance thermometer. A closed loop system featuring a heating/cooling unit with short reaction time and accurate temperature measurement controls the SST. In constant performance mode, full thickness pig skin and human skin showed no macroscopically visible alteration in surface appearance. In varying mode, the 24-h *in vivo* circadian SST rhythm (Lorenz et al., 2019) was simulated *in vitro* in less than 1 hour by changing the temperature in the heating/cooling element accordingly.

In general, human skin showed a fast response to temperature increase or decrease, whereas full thickness pig skin exhibited a time lag. Performance qualification on human skin included infinite dosing of water tempered to 15°C or 6°C. In both cases, a fast on-set and incline similar to a saturating exponential was observed. Cooling of the skin surface with tap water demonstrated lower extent (AUC) and maximum temperature decrease ( $\Delta T_{max}$ ) than with water at a temperature of 6°C. Both parameters were significantly different for the two different temperatures.

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**Presentation:** Poster



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## Assessment of cooling effects of topical products on human skin *in vitro*

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In wound healing, topical gel products support moisture and pH-value of small superficial wounds and dexpanthenol sprays are recommended for slight burns and sunburn. Some products claim an auxiliary cooling effect. Pursuant to 3R, we evaluated the impact of selected wound gels and dexpanthenol sprays on the extent (AUC) and maximum temperature decrease ( $\Delta T_{max}$ ) on dermatomized human skin *in vitro* under in-use conditions.

Skin Surface Temperature Modulation and Control (Skin-Temp-MC) device features a contact-less digital IR and a miniature resistance thermometer and allows for fast and accurate monitoring and controlling the skin surface temperature. After equilibrating the skin surface temperature to 32°C, controls and topical products were evaluated. IR thermometer was used for gels and water as first aid in burns care (Cho and Choi, 2017; Venter et al., 2007). Contact thermometer was used for spray experiments. Controls included tap water (15°C) and cooled water (6°C) under infinite-dose conditions, wound gels and topical sprays under finite-dose conditions.

Three different cooling curve shapes were observed: 1) fast on-set and incline similar to a saturating exponential, 2) falling and rising curve progression (rectangular function), and 3) no or negligible effect.

Two parameters AUC and  $\Delta T_{max}$  enable direct comparison of water controls, gels and sprays. Tap water was successfully integrated as positive control, AUC and  $\Delta T_{max}$  were significantly different for both temperatures with the cooling effect being greater for the colder water. Bepanthen® Kühlendes Schaumspray demonstrated a pronounced cooling, the effect of Panthenol® Spray was very low and statistically different for AUC and  $\Delta T_{max}$ . Add on, Bepanthen® Kühlendes Schaumspray was quickly absorbed by the skin, whereas Panthenol® Spray remained as foam film on the skin surface. Gels BepanGel® Wundgel (Zhang et al., 2020), octenisept® Gel and Tyrosur® Wundheilgel were superior to MediGel® Schnelle Wundheilung in terms of cooling (AUC and  $\Delta T_{max}$ ).

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**Presentation:** Poster

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## Human-relevant approaches to assess eye corrosion/irritation potential of agrochemical formulations

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Multiple *in vitro* and *ex vivo* eye irritation and corrosion test methods are available as internationally harmonized test guidelines for regulatory use. Despite their demonstrated usefulness to a broad range of substances, they have not been widely adopted for testing agrochemical formulations due to a lack of concordance with results from the rabbit eye test. The inherent variability of the rabbit test, differences in the anatomy of the rabbit and human eyes, and differences in modeling exposures in rabbit eyes relative to human eyes contribute to this lack of concordance. Ultimately, the regulatory purpose for these tests is protection of human health; therefore, there is a need for a testing approach based on human biology. This presentation reviews the available *in vivo*, *in vitro* and *ex vivo* test methods with respect to their relevance to human ocular anatomy, anticipated exposure scenarios, and the mechanisms of eye irritation/corrosion in humans. Consideration of the mechanisms of eye irritation, and the strengths and limitations of the methods show that the *in vitro/ex vivo* methods are as or more reflective of human biology and less variable than the currently used rabbit test. Combining structural and functional information about a test substance with results from human-relevant methods will ensure the best protection of humans following accidental eye exposure to agrochemicals.

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**Presentation:** Poster



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## Assessment of skin sensitization potential of fragrance ingredients using the U-SENS<sup>TM</sup> assay

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Ethical considerations and the European Union's legislative ban of animal testing on cosmetic products has accelerated the development of several *in vitro* methods to characterize the skin sensitization potential of chemicals. The U-SENS<sup>TM</sup> assay was developed to address the third key event of the skin sensitization adverse outcome pathway (AOP) (OECD, 2012), dendritic cell (DC) activation, and is described in OECD test guideline 442E (OECD, 2017). U-SENS<sup>TM</sup> quantifies the change in the expression of a cell surface marker, CD86, associated with monocyte and DC activation in the human histiocytic lymphoma cell line U937. A dataset of 68 fragrance ingredients comprising of 8 non-sensitizers and 60 sensitizers was tested in the U-SENS<sup>TM</sup> assay. We aimed to determine how well U-SENS<sup>TM</sup> predicted the sensitization potential of fragrance ingredients when compared to weight of evidence (WoE) from combined historical human and animal data. Of the non-sensitizers, 3 were predicted to be negative while 5 were predicted to be positive. Of the sensitizers, 49 were predicted to be positive, while the remaining 11 were negative. Positive and negative predictive values were 91% and 21%, respectively. No specific chemical property (e.g., solubility and interference) could account for the misclassified ingredients. As a general agreement within the scientific community, that one single NAM would not be sufficient to replace the animal-based methods for skin sensitization, combining complementary *in silico* and *in vitro* methods to the U-SENS<sup>TM</sup> data should be integrated to define the hazard classification of fragrance ingredients and therefore perform the risk assessment.

**Presentation:** Poster

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## An organotypic reconstructed oral mucosa model enabling the study of host-microbiome reactions shows multi-species biofilm promotes human gingiva epithelial barrier function

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The healthy oral mucosa maintains an ecological balance with abundant microbes. It constantly adapts itself to the changing microbial exposure to maintain homeostasis. However, there is little direct evidence supporting this, as most of the current knowledge is from animal studies and simple monolayer cells which are less human representative. The aim of this study was to develop an *in vitro* co-culture model which closely resembles the interplay between the healthy mucosa and the microbiome, to determine whether microbes contribute to the oral barrier function, e.g., a highly proliferative, extensively stratified, thick and antimicrobial defense primed epithelium, with the support of fibroblasts underneath. Two optimal models were used: a 3D reconstructed human gingiva (RHG), and a multi-species biofilm representing commensal microbiota. The organotypic RHG consists of a stratified gingiva epithelium on a fibroblast-populated hydrogel (mimicking lamina propria). Biofilm was cultured from healthy human saliva and consists of typical commensal genera *Granulicatella* and major oral microbiota genera *Veillonella* and *Streptococcus*. Biofilm was applied topically to RHG and host-microbe interactions were studied over 7 days. Compared to the unexposed RHG, biofilm-exposed RHG showed increased epithelial thickness, more organized stratification and increased keratinocyte proliferation. Furthermore, biofilm exposure increased the production of RHG anti-microbial proteins Elafin and HBD2, as well as the transcription of Elafin, HBD2 and HBD3 but not HBD1, adrenomedullin or cathelicidin LL-37. Inflammatory and antimicrobial cytokine secretion (IL-6, CXCL8, CXCL1, CCL20) showed an immediate and sustained increase indicating protective host response. In conclusion, exposure of RHG to commensal oral biofilm actively contributes to the RHG epithelial barrier function. Such a human-representative model has huge potential for studying the dy-



dynamic host-microbe interactions in the oral cavity, thus providing a valuable platform for further studies, e.g., the effect of oral-administrated cosmetics, health care products and medication which may influence the healthy host-microbiome balance.

**Presentation:** Poster

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## There is always an alternative. A thought-starter to stimulate and organize discussion of science- policy needs for a sustainable regulation of chemicals

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The success of the European Green Deal and its Chemicals Strategy for Sustainability (European Commission, 2020) will depend on Green Chemistry. The latter implies the need to improve the safety of chemical processes, avoid waste, use renewable feedstock, improve the energy footprint, and change to safe and sustainable chemicals and products. For safety and sustainability, Green Chemistry needs Alternative Methods (Worth, 2020), i.e., Non-Animal Methodologies (NAMs), but it may also profit from an alternative socio-economy reducing the overall exposure to chemicals and alternative regulatory procedures. Here we draw a mind-map, outlining the hypothesis, that a Green Chemistry policy can lead to a society, in which there is always an alternative to animal testing, be it NAMs, or alternatives to the use of pesticides, biocides, chemicals and pharmaceuticals, or alternative regulatory assessment processes requiring comprehensive, green chemistry and NAM toxicology datasets for sustainable uses. For the success of Green Chemistry and alternatives to animal testing, an international science-policy dialogue may also be essential as well as the development of indicators to set goals and monitor the transition to sustainability. This may include progressive, utopian ethical and socio-economic policy discussions as drivers for longer term developments. Examples concern the need to take seriously the conflicts from empathy and ethical arguments for animal protection and the need to overcome current economic growth constraints to achieve true human wellbeing. In addition, targeted education may be essential. We are inviting congress participants to extend our mind-map for a collective understanding and engagement with all stakeholders.

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**Presentation:** Poster

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## Pharmacokinetic functions of human induced pluripotent stem cell-derived small intestinal epithelial cells

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To develop a novel intestinal drug absorption system using intestinal epithelial cells derived from human induced pluripotent stem (iPS) cells, the cells must possess sufficient pharmacokinetic functions. However, the CYP3A4/5 activities of human iPS cell-derived small intestinal epithelial cells prepared using conventional differentiation methods is low. Further, studies of the CYP3A4/5 activities of human iPS-derived and primary small intestinal cells are not available. To fill this gap in our knowledge, here we used forskolin to develop a new differentiation protocol that activates adenosine monophosphate signaling. mRNA expressions of human iPS cell-derived small intestinal epithelial cells, such as small intestine markers, drug-metabolizing enzymes, and drug transporters, were comparable to or greater than those of the adult small intestine. The activities of CYP3A4/5 in the differentiated cells were equal to those of human primary small intestinal cells. The differentiated cells had P-glycoprotein and PEPT1 activities equivalent to those of Caco-2 cells. Differentiated cells were superior to Caco-2 cells for predicting the membrane permeability of drugs that were absorbed through a paracellular pathway and via drug transporters. In summary, here we produced human iPS cell-derived small intestinal epithelial cells with CYP3A4/5 activities equivalent to those of human primary small intestinal cells.

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## National survey on the protocol review guide applying the 3Rs principles in Korea

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Based on the 2019 annual government report, 410 Institutional Animal Care and Use Committees (IACUCs) were registered with the Animal Protection & Welfare Division of the Animal and Plant Quarantine Agency, Ministry of Agriculture, Food and Rural Affairs. Korea IACUCs are classified as four different types: hospitals (35), government (73), academia (126), and industry (176). The authors developed Korean Guides, to provide consistent application of animal study protocol templates, simplifying effective and efficient protocol review incorporating Korean laws and required regulations: “Guide for the Animal Study Protocols” and “Guide for the IACUC Lay Member”. This project was funded by the Animal and Plant Quarantine Agency and the Bioethics Information Centered (BIC) Study. A national survey was conducted from 22 September to 20 October 2020, to determine the views of three specific groups on the draft guides and protocol templates, namely: IACUC members, IACUC administration staff, and researchers. Three different types of questionnaires were prepared, according to the requirements for each specific group, with a link to the online version. A supporting email to all IACUC administration staff was distributed by the funding government agency. A total of 162 respondents participated: 43 IACUC members, 50 IACUC administration staff, and 69 researchers. The results were analyzed by professional survey consultants and the findings informed the final version of the two Guides, published in December 2020. The funding government agency is also shared the findings of the survey with those directly involved with the care and use of animals for scientific purposes, i.e., institutional managers, investigators, IACUC and staff members, animal carers, as well as animal advocacy groups and those involved with policy and legislation. This paper provides an assessment of the overall processes within the Korean research community, as well as a number of the issues and recommendations for improvement.

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**Presentation:** Poster

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## Cytotoxicity of reference electrodes in microphysiometric systems revisited

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Microphysiometric systems offer alternative approaches to animal experiments in various applications (Brischwein and Wiest, 2019). Reference electrodes are usually required to implement electrochemical microsensors in such systems (McConnell et al., 1992). To investigate the cytotoxic properties of silver-based electrodes in microfluidic systems, we performed a biocompatibility assay based on contact inhibition (Wiest, 2016) and compared the cytotoxic effect of a silver wire coated with silver chloride to that of a bare silver wire. We found that a zone of cytotoxicity developed around both wires. Remarkably, the silver chloride coated silver wire produced a larger cytotoxic zone than the bare wire after the same elapsed time. The larger cytotoxic zone is probably due to the fact that the silver chloride coating emits Ag<sup>+</sup> ions with a higher flux than the pure metallic silver surface. A possible explanation is that in silver chloride the silver is bound in the form of an ionic bond, and in silver as a metal bond. Another explanation would be a manufacturing-induced increase in the roughness of the silver chloride layer and the associated larger interfacial surface area. We modelled the ob-



served propagation of cytotoxic zones in a numerical simulation to predict them in fluidic systems. How the observed difference between the two effects is divided cannot yet be distinguished and is subject of further research. This includes investigation of possible other influences such as cellular sensing properties.

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**Presentation:** Poster

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## The E-Morph assay: Screening for anti-/estrogenic substances based on quantitative changes in cell-cell contact organization of breast cancer cells

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Adverse health effects caused by the unintended exposure to endocrine disrupting chemicals (EDCs) including substances with anti-/estrogenic activity in the environment, food, and consumer products are of high public concern. Exposure to EDCs that interfere with normal estrogen hormone function can lead to impaired development, reproduction, and carcinogenesis. The traditional *in vivo* studies for the identification and characterization of EDCs are time consuming, expensive, and require high numbers of test animals. In view of the up to 800 substances that have been listed as suspected EDCs in recent years and the more or less unlimited number of possible co-exposure scenarios, modern cell-based high-throughput screening (HTS) approaches are increasingly playing a central role in regulatory toxicology.

The E-Morph Assay is a novel *in vitro* screening assay that addresses a human-relevant functional endpoint, i.e., the estrogen-dependent reorganization of cell-cell contacts in breast cancer cells (Bischoff et al., 2020; Kornhuber et al., 2021). We used this assay to screen a library of 440 toxicologically relevant industrial chemicals, biocides and plant protection products that were proposed to act on nuclear hormone receptors (estrogen receptor, androgen receptor, glucocorticoid receptor and thyroid-stimulating hormone receptor). The E-Morph Assay detected known estrogenic substances with relative potencies that correlated very well with the ER bioactivity score published by the U.S. EPA Endocrine Disruptor Screening Program. Moreover, the E-Morph Assay identified substances with yet undescribed estrogenic activity, and the two known anti-estrogens Tamoxifen and Raloxifene. These results provide a proof-of-concept study demonstrating the applicability of the E-Morph Assay in cell-based HTS approaches.

A European and international (PCT) patent application for the E-Morph Assay has been filed at the European Patent Office (Schönfelder et al., 2019).

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## Building confidence in using new approach methodologies for consumer-based risk assessment: Challenges and future perspectives

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Using the risk assessment of 0.1% coumarin in a face cream and body lotion as an exemplar case study, we recently demonstrated how new approach methodologies (NAMs) can be applied in Next Generation Risk Assessment (NGRA) to assess the safety of consumer product ingredients (Baltazar et al., 2020). While this study helps build confidence in the use of NAMs for consumer-based



risk assessments, there is an on-going need to demonstrate that these approaches can be used to define low-risk consumer exposures for a wider range of chemicals, exposure scenarios and endpoints. In general, the use of NAMs in NGRA are critically dependent on being able to integrate computational modelling approaches with *in-vitro* assay data in a robust manner. This encompasses a range of challenges, including that the assay data and associated metadata are generated in accordance with appropriate quality standards and are reproducible; that uncertainties in model inferences can be quantified using appropriate computational approaches; that both the models and assay designs undergo relevant forms of evaluation. In order to illustrate and discuss these issues further, we will draw on specific examples from our recent work on evaluating NAMs for use in systemic toxicity and skin allergy. The intention is that these will help illustrate the future challenges that need to be addressed in order to ensure the wider acceptance of NGRA for consumer-based risk assessment.

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**Presentation:** Poster

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## An *in-vitro* model of alveolar inflammation on a breathing lung-on-chip

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The rising need for alternative methods to animal testing in the context of inhaled drug development and pulmonary disease modeling is leading to the development of organ-on-chip systems with complex cellular *in vitro* models. Traditional *in vitro* models lack complex structural and dynamic environment as well as cellular diversity, which are essential to precisely emulate pathological conditions and predict drug response. We aim to develop an advanced and robust *in vitro* model of the human alveolus based on the organ-on-chip developed by AlveoliX AG (Stucki et al., 2018), which allows for physiological stretch and aerosol exposure within the Vitrocell<sup>®</sup> Cloud System.

The human air-blood-barrier in the alveoli is the largest epithelium in direct contact with air, making it a significant way of entry for both pathogens and pharmaceutical agents. To gener-

ate an *in vitro* human model of the air-blood-barrier, we co-cultured alveolar epithelial cells (hAELVi cell line; Kuehn et al., 2016) with monocyte-derived macrophages (differentiated THP-1; Kletting et al., 2018) on the organ-on-chip. The co-culture was subjected to dynamical stretch, emulating the physiological compression and expansion of the alveolar surface during breathing. Additionally, to model alveolar inflammation and anti-inflammatory drug administration, the co-cultures were subjected to different inflammatory stimuli, like lipopolysaccharide, and the anti-inflammatory drug Budesonide, respectively. Subsequent cytokine release from the co-cultures was analyzed by bead-based flow cytometry. Transepithelial electrical resistance and paracellular permeability were measured to monitor barrier integrity in static and breathing-like conditions. Interestingly, the intensity of inflammatory response seems to be modulated by the breathing motion and is weaker than in static conditions, highlighting the importance of dynamics in modeling the air-blood-barrier *in vitro*. Treatment with Budesonide mitigated cytokine release in both static and dynamic conditions.

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**Presentation:** Poster

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## GARD<sup>™</sup>skin and GARD<sup>™</sup>potency: A proof of concept study to investigate the applicability domain for agrochemical formulations

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Several *in vitro* methods for detection of skin sensitization have been formally validated for regulatory use. The Genomic Allergen Rapid Detection (GARD<sup>™</sup>) is a genomic based assay plat-



form which is currently being assessed for inclusion in the OECD test guideline program. GARDskin and GARDpotency, address Key Event 3 (dendritic cell activation) of the skin sensitization Adverse Outcome Pathway, and provides reliably potency information for several chemical classes.

The purpose of this work is to verify the applicability domain of GARDskin and GARDpotency, for the product class of agrochemical formulations to favor future regulatory uptake.

For this proof of concept, 20 agrochemical formulations were tested using GARDskin. When GARDskin was positive, GARDpotency assay was used to determine the severity of sensitization potential. Tests were conducted according to the assay developer Standard Operating Procedures. The selected agrochemical formulations were liquid (11 water-based; and 9 organic solvent-based) with a balanced distribution (11 not classified; 7 GHS cat 1B; 2 GHS cat 1A, which is rare for agrochemical formulations). GARD results (available for 18 formulations at this time) were compared with *in vivo* data (mouse LLNA) already available for registration purpose, in order to verify concordance (GHS hazard and potency categories). For hazard, GARDskin was able to correctly identify 7/10 not classified (true negatives) and 7/8 GHS1B/1A (true positives), with 1 false negative and 3 false positives. The accuracy, sensitivity, and specificity for prediction of hazard were 77.8% (14/18), 87.5% (7/8) and 70.0% (7/10), when using available LLNA results as classification reference. Additionally, GARDpotency was able to correctly identify 5 GHS cat 1B and 1 GHS cat 1A out of 7 correctly predicted sensitizer (underprediction from 1A to 1B occurred in 1 case).

In conclusion, GARDskin and GARDpotency, showed a satisfactory performance in this initial proof of concept.

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## Dog as the experimental model: Laboratory uses of dogs in the United States

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Dogs are used in all types of biomedical research, from behavioral to invasive surgical procedures, often resulting in pain and distress to the animals and, ultimately, in their death. While the literature reporting research studies using dogs is extensive, there are few reviews focusing more broadly on the use of dogs in biomedical research. Therefore, NAVS undertook a study to investigate how dogs are currently being used in invasive experiments in the United States.

Data on the extent of invasive biomedical procedures on dogs in U.S. laboratories were accessed across several key sources of information, including research publications, National Institutes of Health (NIH) grant proposals, and United States Department of Agriculture (USDA) data.

Publication and grant data indicated the largest number of invasive biomedical procedures using dogs involved some type of cardiovascular research, although the number of dogs per study was typically not large. Another significant category for dog use, drug/regulatory studies, often involved a greater number of dogs. Certain data categories were susceptible to missing or incomplete information, including dog number, pain management, dog source, and disposition of dogs. Justifications for selecting dog as the animal model were usually provided, although consideration of alternatives was not. USDA data provided information about laboratory uses of dogs by USDA-licensed institutions, including number, cost, and sources of dogs; number of painful procedures; and Animal Welfare Act violations.

Results of this study will be used to inform and support initiatives to reduce, refine, and replace dogs in invasive biomedical research. They may also benefit parallel initiatives such as the recent recommendation from the National Academies of Sciences, Engineering, and Medicine for the U.S. Department of Veterans Affairs to collaborate on identifying alternatives.<sup>1</sup>

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**Presentation:** Poster

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## A study on the correlation between the HET-CAM and the reconstructed vaginal tissue model for safety test of feminine wash

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For assessing vaginal irritation of feminine wash, the rabbit vaginal irritation test (RVI) has been the most frequently used. However, since March 2009, using animal for testing cosmetics has been banned by the council of the European Union, and also has been banned in Republic of Korea since February 2017. According to the trend of animal testing ban, many alternative animal test



methods for testing safety of personal care products and ingredients have been developed. Cell-based models, reconstructed tissue models and explant-based models are a typical *in vitro* test for assessing irritation of feminine wash. These have been confirmed that they have the potential to replace the RVI.

Hen's egg-chorioallantoic membrane (HET-CAM) assay has been used as an alternative method to the Draize rabbit eye irritation test. And it is now widely used. According to ISO 10993-10, it is suggested that irritation potential for eye and vaginal membrane is similar (Palmeira-de-Oliveira et al., 2018).

In this study, the correlation between the HET-CAM and the reconstructed vaginal tissue model in vaginal irritation test of feminine wash was studied. It was tested in various products, such as gel and bubble types. Test results showed different levels of irritation. These results were similar in the two test methods and were able to distinguish between severely and weak irritating products. The R2 between the two methods was 0.6164 for gel types, 0.7117 for bubble types and 0.6846 for total products. We have confirmed the possibility of HET-CAM as a test method for vaginal irritation test through a comparative study of the HET-CAM and the reconstructed vaginal tissue model. The HET-CAM has the advantage of saving time and cost compared to other vaginal irritation tests (RVI, the reconstructed vaginal tissue model).

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**Presentation:** Poster

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## Identification of skin sensitizing impurities in reaction mixtures by fluorescent nitrobenzoxadiazole-labeled glutathione

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Allergic contact dermatitis is a critical issue in the development of new chemicals. Even when exposed to minor impurities with strong skin-sensitizing properties, allergic contact dermatitis can be induced. However, it is very difficult to identify them in product mixtures. In this study, in order to identify very small amounts of skin sensitizers in mixtures, we designed fluorescent nitrobenzoxadiazole-labeled glutathione (NBD-GSH) and developed a new method to detect and identify them as NBD-GSH adducts with high sensitivity by liquid chromatography (LC) with fluorescence detection and mass spectrometry (MS).

When twelve skin sensitizers and three non-sensitizers were reacted with NBD-GSH, adducts formed only with the skin sensitizers and LC-MS analyses showed that NBD-GSH reacted with the skin sensitizers via its thiol and amino groups. Using 1-chloro-2,4-dinitrobenzene known as a strong skin sensitizer, the limit of detection (LOD) of an NBD-GSH adduct was determined as the concentration of  $6 \times 10^{-8}$  mol/L in a sample solution by LC with fluorescence detection. When primary alcohol was oxidized and the reaction mixture was incubated with NBD-GSH, an NBD-GSH adduct formed with the trace level of skin-sensitizing aldehyde which was produced as an intermediate between the corresponding alcohol and carboxylic acid, and could be specifically detected by fluorescence detector and identified by LC-MS. We will demonstrate that this method will be useful for detection and identification of small amounts of skin sensitizers in raw materials, intermediates, reaction mixtures, and end products in the chemical industry.

**Presentation:** Poster

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## Pyrogen detection in pharmaceutical quality control: Moving away from the rabbit pyrogen test with a ready-to-use monocytic cell line

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The batch release of parenteral drugs relies on several quality control tests including the pyrogen test. Currently, the standard test is the rabbit pyrogen test (RPT), which has been widely used for decades despite known weaknesses.

After the discovery of the reaction of Limulus Amebocyte Ly-sate (LAL) towards endotoxin (a very potent pyrogen coming from Gram negative bacteria) the LAL test was progressively adopted by the pharmaceutical industry, as a replacement of the RPT for batch release.

If the RPT can measure the actual pyrogenic reaction in rabbits, the LAL test can only detect the presence of one pyrogenic contaminant (lipopolysaccharide, LPS) in a sample, leaving the risk to miss out on other pyrogenic contaminants, often mentioned as non-endotoxin pyrogens (NEPs). It is also worth mentioning that the LAL test highly relies on the use of an endangered animal species.

In the 90s, the first monocyte activation test (MAT) was patented, opening a new way for *in vitro* pyrogen tests. MAT brings several advantages: it is a test that mimics the human reaction to pyrogens, thanks to the release of cytokines by monocytic cells. Recently, ready-to-use solutions have been developed to facilitate the implementation of MAT in pharma-



ceutical quality control laboratories, including a test system relying on the use of a ready-to-use monocytic cell line, the Mono-Mac-6 cell line.

Although MAT has been accepted as a compendial method in the European Pharmacopeia for more than 10 years, its adoption has remained slow. One of the concerns relates to the ability of monocytic cell lines to detect NEPs.

We have demonstrated that the MM6 cells are suitable for the detection of various NEPs targeting different monocytic toll-like receptors, making MM6 cell line-based MAT suitable for RPT replacement.

**Presentation:** Poster

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## Assessing experimental uncertainty in defined approaches for skin sensitization

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Prediction models of most toxicological assays translate continuous data to a binary classification (“positive” or “negative”) using cut-off values. Mostly these cut-offs do not consider data variability. Some OECD test guidelines, however, provide a range close to the cut-off. If a test result is in this range, a repetition of the test is proposed. Yet, these ranges were based on few data and not systematically derived. In the present study we determined the borderline ranges from multi-laboratory ring trial studies for five non-animal methods addressing skin sensitization: Direct Peptide Reactivity Assay (DPRA, OECD TG 442C), KeratinoSens<sup>®</sup> and LuSens (OECD TG 442D), and human cell line activation test (h-CLAT, OECD TG 442E) as well as the kinetic direct peptide reactivity assay (kDPRA; draft update of OECD TG 442C). We used a uniform statistical approach based on the log median absolute deviation (MAD). Implementing the proposed borderline ranges helps to assess certainty of both individual test result and of combinations of multiple data sources in a defined approach (DA) or an integrated approach. The draft OECD guideline on DAs for skin sensitization provides the first regulatory application of borderline ranges to the “2 out of 3” DA.

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**Presentation:** Poster

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## Cytotoxicity evaluation of tetrabromobisphenol A and polystyrene nanoplastics on RTgill-W1 fish cells

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Brominated flame retardants (BFRs) and nanoplastics (NPs) are contaminants of emerging concern due to their persistent and ubiquitous presence in the environment. Most of these compounds can be bioaccumulated through the food chain, potentially causing detrimental effects on human and environmental health. Particular interest is now focused on the ability of NPs to interact with other chemical pollutants which might increase the toxic effects in aquatic organisms.

This work evaluates the cytotoxic effects of tetrabromobisphenol A (TBBPA), one of the most prevalent BFR, and polystyrene nanoparticles (CML Latex Beads, 40 nm) in a rainbow trout gill epithelial cell line (RTgill-W1), after individual and combined exposures for 24 hours. Different culture media compositions were tested to compare their influence on cytotoxicity: L15 with 10% serum, L15 with 2% serum, and L15/ex. Three complementary endpoints were used to assess metabolic activity (Alamar Blue, AB), and integrity of plasma membrane (5-Carboxyfluorescein Diacetate Acetoxymethyl Ester, CFDA-AM), and endosomal compartment (Neutral Red Uptake, NRU). After evaluating single exposures in concentrations ranging from 1 to 150 µM for TBBPA (0.27-108.8 µg/mL) and from 0.1 to 200 µg/mL for NPs, relevant conditions were selected for co-exposures. In addition to the cytotoxicity evaluation, further experiments were conducted, including alkaline comet assay for potential genotoxicity, perturbations of transepithelial resistance (TEER), and morphological evaluation of cell cultures.

Our results suggest that both TBPPA and NP have detrimental effects on the viability of RTgill-W1 cells under our experimental conditions, both individually and in combination. The toxic injury is highly dependent on the composition of the exposure media, being metabolic activity the most sensitive endpoint. The



presence of serum in the culture media influences the behavior of the polystyrene nanoparticles, inducing aggregation and higher nanoparticle sizes, which could partially explain the observed effects.

**Presentation:** Poster

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## Increasing the availability and quality of human tissue for research

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Advances in 3D and other *in vitro* tissue model systems have led to important developments in research on human diseases, advancement of novel therapies, and safety testing. In addition, histological and cellular studies of human tissues continue to serve as keystones in understanding disease and health processes. Technologies, like organs-on-chips and 3D bioprinting, rely on human cells and have the potential to have a powerful impact on scientific advancement. As interest grows in using more human tissues, guidelines are needed to encourage consensus among stakeholders concerned with the use of human tissues and human-model systems, to establish and maintain best practices, to promote effective quality control systems, to facilitate education and training, to support journal editors, and to help end users and regulators who need to understand results and draw conclusions based on human tissue data. A lack of a standardized approach to the donation, procurement, and processing of tissue, coupled with the absence of a U.S. wide strategy to oversee recovery and use leaves gaps in tissue quality and availability. Our working group, comprised of stakeholders from the research community, regulatory agencies, and organ procurement organizations, are exploring and addressing the barriers to the use of human tissues and cells in research. We have established a set of recommendations and are developing guidelines for standardizing the characterization of human tissues and cells to facilitate greater access to high-quality human tissues for biomedical research and toxicology and help ensure the transition away from the dependence on animal models.

**Presentation:** Poster

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## Immunogenicity studies of pre- and post-exposure anti-rabies vaccination (ARV) using BALB/c mice: A systematic review

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Vaccination against rabies is an effective control measure of the disease. Immunity development following ARV needs optimization of various immune parameters. Those parameters were tested initially in laboratory animal models before their testing in large animals. We conducted a systematic review (SR) of studies performed on laboratory animal models to assess immune parameters that affect the immunity development following ARV.

PubMed, Web of Science and SCOPUS were searched for relevant publications until 14.06.2020. Relevant publications were selected based on pre-determined inclusion/exclusion criteria by two independent reviewers.

Our search revealed that BALB/C mouse is the mostly used model in this regard. Five studies which used BALB/C mice commencing from year 2000 until the search date were selected for this paper. Except one, all studies have used female mice. Age group varied from 4 to 8 weeks. All studies have used plasmid/DNA vaccine constructs and immunity level was determined by measuring rabies virus neutralizing antibody titers (VNA).

Based on the review, most effective route of vaccination was intramuscular (IM) with the dose of 100 µg/100 µl of the vaccine construct. Using doses > 100 µg, provided no advantage in the IM route. In all studies, IM immunization have shown > 50% protection after the post-vaccination virus challenge. In post-exposure studies, DNA vaccine constructs had triggered the production of rabies-specific antibodies as rapidly as cell culture-based vaccines and protection from the challenge were > 50%, even with a single dose. A study showed gene-gun delivery method for the immunization had been effective in producing a VNA titer of 8 IU/ml but was less than with IM vaccination (VNA = 16 IU/ml). Another study showed the effectiveness of administering comparatively higher doses of oral vaccines which significantly increased VNA titers with > 50% protection following the challenge.

In the conclusion, BALB/C mice seems to be an effective screening model for immunogenicity following ARV.

**Presentation:** Poster



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## Non-animal derived antibodies in research and diagnostics

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The most widely used *in vitro* technology for antibody selection is antibody phage display (Breitling et al., 1991). This method allows to select recombinant monoclonal antibodies directly from human naïve or patient derived antibody gene libraries, hence without any need for animal immunization. These antibodies are uniquely identified by their DNA sequence, which can be used as starting material to indefinitely produce the corresponding antibody molecule, but that can also be modified at need to generate a reagent that better fits the intended final application. Nowadays, phage display for antibody selection is already a standard for therapeutic human antibody generation, a field where the unique features of recombinant antibodies are widely explored. Recently, the applicability of this technology also for diagnostics and research has been recognized and encouraged by the EU (Viegas Barroso et al., 2020), in line with the Directive on the protection of animals used for scientific purposes (2010/63/EU).

In this presentation we will illustrate the potential of the *in vitro* technology for antibody selection in comparison to the most known immunization approaches. We will provide examples of the applicability of the method to replace animal sera in diagnostics and research (recombinant Multiclonal secondary antibodies) and a glimpse on the positive impact that antibody format conversion and modifications can have, likewise in therapy, also on diagnostic applications.

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**Presentation:** Poster

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## Generation of reporter-gene models based on genetically engineered progenitor cells using CRISPR-Cas9 technology

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CRISPR-Cas9 has become a very popular gene-editing technique and its potential applications, including in the field of toxicology, are endless.

We present a new approach for generating reporter-gene models based on genetically engineered progenitor cells, which could make toxicity assessment of whole aerosols *in vitro* faster. We focused on the stress-induced protein heme oxygenase 1 (HMOX1) and tagged it with green fluorescent protein (GFP) by CRISPR-Cas9-mediated knock-in. With the induction of the fusion protein being driven by the endogenous promoter of the target gene, such reporter models allow easy monitoring of stress responses upon treatment with test compounds.

The method was first established in the immortalized cell line HEK293, in which five different guide RNAs (gRNAs) were screened. Four of them targeted an area close to the start codon of HMOX1, while the last one targeted an area close to the stop codon. The cells were transfected by electroporation with CRISPR-Cas9, the gRNA, and a DNA repair template containing the GFP gene surrounded by homology arms. Successfully genetically engineered clones were identified by PCR, western blot, and whole-genome sequencing. Two gRNAs gave rise to positive clones in which HMOX1 was tagged at the N- or C-terminal ends. The inducibility of the fusion protein was tested upon treatment with hemin, a well-known oxidative stress inducer and substrate of HMOX1. We observed that HMOX1-GFP was induced in a dose-dependent manner upon hemin treatment. Preliminary results indicated that the system responds to exposure to aerosol fractions.

The optimized technique will then be applied to primary airway epithelial cells. These cells grow and differentiate at the air-liquid interface, forming three-dimensional organotypic tissues, and can be directly exposed to whole aerosols. Subsequent measurement of the fluorescence emission will allow us to quickly evaluate aerosol toxicity in close-to-physiological conditions.

**Presentation:** Poster

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## Reconstruction of hepatic sinusoidal zonation by organ-on-a-chip from micronit

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The demand for developing humanized *in vitro* test for the safety evaluation of chemical substances is increasing in recent years to reduce the number of animal test. Based on such demands, the development of Microphysiological Systems (MPS), which culture cells in a microenvironment similar to that of living organisms, have been actively pursued. In this study, we examined the culture condition that medium is perfused in a sealed compartment to mimic the zonation observed in hepatic sinusoids. Organ-on-a-chip (OOC) (Enschede, The Netherlands), which is commercially available from Micronit, was used as the MPS. After the membrane was coated with type I collagen, human liver cancer-derived cell line HepaG2 were seeded and cultured in static conditions until the cells adhered (4-5 hours). Membrane with adherent cells were immersed in 10 ml of medium in 10 cm dish and incubated for 72 hours. After 72 hours, the OOC chamber containing the membrane was set in a folder and perfusion-culture was started. When the upper and lower chambers were perfused with medium, the cells grew in an island like-shape as in static culture. When medium was perfused only in the lower chamber of membrane, cells formed a uniform monolayer, and the cell morphology was different from that of static culture. The assay using LIVE/DEAD<sup>®</sup> Cell Viability Kit (Thermo Fisher Scientific) demonstrated that the proportion of dead cells increased toward the outlet under the condition perfusing medium in the lower chamber of membrane alone. These results suggest that a concentration gradient in the medium composition mimicking hepatic sinusoidal zonation may have been formed under this culture condition.

**Presentation:** Poster

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## Quality control of hepatic stem cells derived from human fetal hepatocytes using novel nanoparticles

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**Introduction:** The quality control of the stem cells is very important for regenerative medicine and cell-based assay system for drug discovery. In cell transplantation therapy, malignant transformed cells, that are generated in the process of cell culture, proliferate abnormally *in vivo* after cell transplantation and pose a risk of tumor formation. Therefore, it is required to establish a method for eliminating such tumorigenic cells. We have previously found that tumorigenic (transformed) cells emerged during the induction process of normal human fetal hepatocytes (Hc cells) into hepatic stem cells by sodium butyrate (SB) treatment (Kiyota et al., 2007). In this study, we use nanoparticles (hybrid liposomes, HLs) to selectively eliminate tumorigenic cells. HLs had been developed at our university and specifically induced apoptosis for cancerous cells without affecting normal ones (Matsumoto et al., 2005).

**Materials and Methods:** HLs were prepared by sonicating the mixture of vesicular and micellar molecules. The tumorigenicity was evaluated using the soft agar colony forming method. CYP3A4 activity was measured as benzyloxyresorufin-O-dealkylation activity. The cell membrane fluidity was measured by the fluorescence depolarization method. The selective accumulation of HLs containing fluorescent phospholipid was examined by flow cytometric analysis. Induction of apoptosis was detected by measurement of activated caspase-3.

**Results and Discussion:** HLs suppressed the colony forming ability of SB-treated Hc cells. The result that high CYP3A4 activity of the HLs-treated cells was observed in a three-dimensional *in vitro* assay, which induces their differentiation into hepatocytes, indicated HLs treatment did not diminish hepatic stem cells. HLs selectively fused and accumulated into the cells, which increased in cell membrane fluidity. Furthermore, apoptosis was induced for HLs-treated cells. These results indicate that HLs selectively eliminated the tumorigenic cells and seemed to be useful for quality control of the stem cells.

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**Presentation:** Poster

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## Response to recommendation on non-animal-derived antibodies

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In May 2020, the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) published a recommendation on non-animal-derived antibodies (NADA) (Viegas Barroso, 2020), calling for their widespread replacement in basic research, animal health and therapeutics. In response, the European Animal Research Association (EARA), European Federation of Pharmaceutical Industries and Associations (EFPIA) and AnimalhealthEurope surveyed members and established a working group of leading scientists from public and private biomedical institutions to raise their scientific concerns over any immediate obligations to require the use of NADA.

Of 133 members surveyed, 74% reported it would not be possible to completely replace animal-derived antibodies (ADA) at their institution, citing reasons such as lack of availability and the essential role of ADA in some applications. Respondents commended the forward-thinking approach of the report in line with Directive 2010/63/EU, however, were concerned that the recommendation oversimplifies the current situation and did not address the difficulties associated with adopting NADA at this time.

The expert working group expanded on these issues held by the biomedical community in a report published in November 2020, by exploring the scientific justifications for the continued need for ADA in some applications (EARA, 2020). Members of the group represented a wide range of fields and had experience of using both ADA and NADA in research and drug discovery but had reservations about the recommendation. The report focused in particular on the need for ADA in therapeutic and animal-health related fields, which were omitted from the EURL ECVAM report. This presentation will showcase some of the scientific arguments for the continued use of ADA where necessary, and the measures currently taken by public and private research institutions to ensure reduction in the number of animals used.

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bodies, EUR 30185 EN. Publications Office of the European Union, Luxembourg. doi:10.2760/80554

**Presentation:** Poster

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## U-SENS™: APC can be used as an alternative in case of strong chemical-induced autofluorescence at the FITC-specific wavelengths

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Decades of intensive research have provided Adverse Outcome pathways and New Approach Methodologies (NAM) for animal-free assessment of skin sensitization. The U-SENS™ (OECD Test Guideline 442E), addressing the key event 3 of the skin sensitization has been foreseen to be combined with complementary information in defined approaches in the Next Generation Risk Assessment.

The U-SENS™ is modelling the dendritic cell activation upon exposure to chemicals. Upon contact with sensitizers, U937 human histiocytic lymphoma cells are activated and increased the CD86 expression measured by flow cytometry using a fluorescein isothiocyanate (FITC) CD86 monoclonal antibody. The U-SENS™ assay has been shown to be applicable to a broad range of chemicals (fragrances, dyes, preservatives, UV filter, and non-cosmetics ingredients...). However, strong fluorescent chemicals emitting at the same wavelength as FITC might interfere with the flow cytometric detection and thus cannot be correctly evaluated using FITC-conjugated antibodies.

Therefore, the aim of the present study was to 1) identify other fluorochrome-tagged antibodies, 2) demonstrate similar results as the FITC-tagged antibodies by testing the proficiency substances identified in the OECD TG 442E, and 3) determine the outcome of an interfering FITC-chemical.

As an alternative of the fluorophore FITC (excited at 488 nm), the APC (excited at 633 nm) has been evaluated. The results showed that all acceptance criteria of the test method were fulfilled by using the APC fluorochrome. The 10 OECD proficiency chemicals provided similar results (EC150 values and classification) as the FITC-tagged antibody. An enlarged set of 30 non-to-extreme sensitizers confirmed the relevance of this APC fluorochrome. In addition, the applicability domain of the APC was demonstrated with a flavonoid interfering FITC-chemical.

In conclusion, the use of the APC coupled antibody could be seen as an alternative fluorochrome of the FITC-tagged antibodies enlarging the applicability domain of the U-SENS™ test method.

**Presentation:** Poster



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## Developing a defined approach for eye irritation testing

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Regulatory acceptance and implementation of new approach methodologies depend on public-private partnerships, which enable communication and cooperation among federal agencies and the private sector. To that end, the PETA Science Consortium International, the Interagency Coordinating Committee on the Validation of Alternative Methods, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, and CropLife America companies are collaborating to assess the applicability of *in vitro* and *ex vivo* test methods to assess eye irritation potential for agriculture pesticide formulations. The goal is to identify methods most appropriate for human-relevant defined approaches to hazard evaluation that do not use animal data. To date, sixteen formulations have been tested in the bovine corneal opacity and permeability, neutral red release, isolated chicken eye, EpiOcular, and porcine cornea reversibility test methods. Results were compared to the hazard classification assigned based on the *in vivo* rabbit test. For each test method, at least one formulation was discordant with the *in vivo* rabbit classification, but none of the methods yielded discordant results for all tested formulations. Initial results indicate that certain test method combinations may be used to predict *in vivo* outcomes. Additional analyses will focus on physicochemical properties and composition of tested formulations to determine if there are any common features that impact *in vitro* test method performance. These data will be considered in the context of a recent review of ocular anatomy and mechanisms of chemically induced ocular irritation in humans and other species, which supported reduced reliance on comparisons to rabbit data to show the validity of other methods, and instead a focus on human-relevance and assay reliability.

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**Presentation:** Poster

1003

## Thyroid disruption rescue of iodide on a zebrafish vertebrate model

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The zebrafish (*Danio rerio*) vertebrate model is broadly used in both human and aquatic toxicity assessment as an excellent alternative to *in vitro* and *in vivo* models due to their cost-efficiency, small size, rapid development, and homology with mammals.

There is an increasing concern about the environmental impacts of chemicals that can disrupt the endocrine system, which can result in adverse effects on developmental, reproductive, neurological, and/or immune functions in both humans and wildlife. Iodide is crucial for thyroid hormone synthesis and function. Iodide can decrease the response of the thyroid gland to the thyroid-stimulating hormone, suggesting a major role in the negative feedback loop.

The aim of this work was to investigate the potential role of iodide-exposure in modulating the impact of EDCs. For that purpose, Sodium Iodide was co-exposed with two known thyroid disrupting (TD) substances (potassium perchlorate, propylthiouracil) at different concentrations. The screening was performed in a three-phase assay. First, TD chemicals were evaluated using the transgenic line tg(tg:mcherry), which expresses red fluorescence in the thyroid gland under the thyroglobulin promoter. The quantification of the fluorescence intensity allows monitoring *in vivo* the upregulation of the thyroglobulin gene expression as a compensatory reaction to thyroid gland disruption. Second, gene expression analysis was carried out to measure thyroid-related genes (tsh $\beta$ , tg, and tpo). Third, thyroid hormone levels (T4, T3, 3,5-T2, and 3,3'-T2) were quantified by liquid chromatography (LC/HRMS) in whole-embryo extracts. Our data show that both compounds induced an increase of the fluorescence of thyroglobulin in the tg (tg:mcherry) transgenic line, overexpression of thyroid-related genes, and a concomitant decrease of T4 hormone levels. Preliminary data shows that iodide co-exposure reversed the TD effect of potassium perchlorate but not that of propylthiouracil.

The zebrafish offers a sensitive and cost-effective model to screen and evaluate potential EDCs.

**Presentation:** Poster



1004

## OPERA, an open-source and open-data suite of QSAR models

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OPERA is a freely accessible standalone application based on the open-source/open-data concept providing a suite of QSAR models for toxicity endpoints and physicochemical, environmental fate, and ADME properties (Mansouri et al., 2018). OPERA follows the five OECD principles for QSAR modeling to provide scientifically valid, high accuracy models with minimal complexity that support mechanistic interpretation, when possible. Experimental data is thoroughly curated, and chemical structures standardized, prior to modeling. Recent additions to OPERA include models for estrogen and androgen pathway activity, and acute oral systemic toxicity developed through international collaborative projects (Kleinstreuer et al., 2018; Mansouri et al., 2016, 2020). Existing OPERA models are also updated regularly. Recently, models predicting plasma protein binding and intrinsic hepatic clearance, two important ADME parameters for *in vitro* to *in vivo* extrapolation, have been updated with the latest publicly available datasets to improve their predictivity and applicability domain coverage. Models predicting physicochemical parameters such as logKow, water solubility, and vapor pressure have been updated to account for highly investigated groups of chemicals such as polyfluorinated substances (PFAS). OPERA also provides a tool for standardizing chemical structures and its models yield prediction accuracy estimates, applicability domain assessments, and experimental values when available. Technical and performance details are described in OECD-compliant QSAR model reporting format (QMRF) reports. OPERA predictions are available through EPA's CompTox Chemicals Dashboard (<https://comptox.epa.gov/dashboard>) and the National Toxicology Program's Integrated Chemical Environment (<https://ice.ntp.niehs.nih.gov/>). The OPERA application can be downloaded from the NIEHS GitHub repository as a standalone command-line or graphical user interface for Windows and Linux operating systems. It is also provided as Python, C/C++ and Java libraries that can be embedded in other applications. The views expressed in this presentation are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA or any federal agency.

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**Presentation:** Poster

1005

## Variability in the rabbit skin irritation assay

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The *in vivo* rabbit test is the benchmark against which new approach methodologies for skin irritation are usually compared. Guideline *in vitro* methods for assessing skin irritation potential are accepted as partial replacements for the *in vivo* test, but none are capable of classifying moderate and mild irritants. A limiting factor in identifying a complete replacement could be the variability inherent to the subjective scoring of erythema and edema in the rabbit test. This is particularly relevant for mild and moderate irritants, where interindividual differences in scoring are most likely to occur. To characterize the reproducibility of the *in vivo* assay, we assessed variability in study results from substances tested multiple times. We compiled and curated 2624 test records, representing 990 unique mono-constituent substances, each tested at least twice. Methodological deviations from guidelines were noted, and multiple data sets with differing tolerances for such deviations were created. Where possible, primary dermal irritation indices were estimated from the available data and used to classify chemicals according to the U.S. Environmental Protection Agency (EPA) skin irritation classification criteria. Globally Harmonized System (GHS) hazard classifications were extracted from study reports when available. Conditional probabilities were used to evaluate the reproducibility of the *in vivo* method in identification of EPA or GHS hazard categories. Chemicals classified as moderate irritants at least once were classified as mild irritants or non-irritants at least 40% of the time when tested repeatedly. Variability was greatest between mild and moderate irritants, which both had less than a 50% likelihood of being replicated. This analysis indicates that variability



of the rabbit skin irritation test should be considered when evaluating the performance of nonanimal alternative methods as potential replacements.

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**Presentation:** Poster

1006

## Development of an *in silico* platform to assess developmental and reproductive toxicity (DART)

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Assessment of developmental and reproductive toxicity (DART) uses a large number of mammals. It is particularly in this field of toxicology that alternative test methods that follow the principles of the 3Rs (Replacement, Reduction, and Refinement) are highly warranted, to reduce the amount of animal testing. There are many efforts to design alternate assays, whose results are very encouraging. However, a challenge remains in the interpretation of the data. DART is very complex, involving many diverse biological processes and interconnected adverse outcome networks. It is now possible to address this challenge with the recent developments in molecular biology, omics datasets, database interconnectivity, and data science.

We have designed a user-friendly web-based platform that enables complex data integration. For example, the interface can be used starting from chemical(s), via (predicted) DART phenotypes, to adverse outcome pathway, and reversely: from an adverse outcome pathway towards DART phenotypes. Molecular fingerprints are used to compute compound similarity, which allows the comparison of toxicity profiles between similar compounds. Additionally, the interface contains DART phenotypic endpoint data for a wide variety of test systems, to compare OECD DART test data with 3R test methodologies (zebrafish, *C. elegans*, slime mold, and cell-based assays). Cross-species phenotype-to-pathway analysis of toxicity endpoints allows the prediction of evolutionary-conserved pathways affected by compounds.

The use of the interface enables the selection of relevant biological tests for DART assessment and supports the prioritization of chemicals and adverse outcome effects for further investigation. By integrating years of scientific research and knowledge that is available in public resources, we aim to boost the acceptance of alternatives to mammalian DART testing. We thank the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) CRACK IT Challenges programme for funding this work.

**Presentation:** Poster

1007

## Application of defined approaches for skin sensitization to agrochemical products

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The assessment of skin sensitization potential is one of the elements included in international regulatory safety evaluation of pesticides. Several non-animal test methods have been adopted as globally harmonized test guidelines that can help to reduce animal use in these evaluations. No single assay is recommended as complete replacement for existing animal tests, such as the murine local lymph node assay (LLNA). Instead, defined approaches (DAs) that integrate data from multiple methods have been proposed to replace animal use for skin sensitization testing. However, these DAs have been evaluated using mono-constituent substances rather than mixtures or formulations (i.e., end-use products, multi-constituent substances with defined compositions). To fill this data gap, we tested 27 agrochemical formulations using the direct peptide reactivity assay (DPRA), the KeratinoSens™ assay, and the human cell line activation test (h-CLAT). Test data were used to evaluate several rule-based DAs that use these methods for hazard and/or potency categorization of skin sensitization. Balanced accuracy for the DAs for predicting skin sensitization hazard *in vivo* ranged from 56% to 73%. The best performing DA for GHS potency classification had a correct classification rate of 52%. By comparison, of the individual test methods, KeratinoSens had the highest performance for predicting *in vivo* hazard outcomes (balanced accuracy = 81% vs. 62% for DPRA and 56% for h-CLAT) and had higher balanced accuracy than any of the DAs. These results demonstrate that non-animal test methods have promising utility for evaluating the skin sensitization potential of agrochemical



formulations. Further investigation will be required to determine whether DAs can outperform individual assays such as KeratinoSens for predicting *in vivo* sensitization hazard of pesticide formulations in general.

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**Presentation:** Poster

1009

## Toxicity of the aerosol ingredient aluminium chlorohydrate (ACH) in an *in vitro* model of human alveolar cells

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Although exposure to most cosmetics is limited to dermal contact, inhalation exposure is reasonably foreseeable when using cosmetic aerosols. Thus, potential adverse effects in the respiratory tract should be investigated to provide a more robust risk assessment of these products (Rothe et al., 2011; Steiling et al., 2014). Aluminium chlorohydrate (ACH) is extensively used in aerosols as the active ingredient of antiperspirants (Schwarz et al., 2018), and animal studies have demonstrated its potential to deposit and induce local toxic effects in the lungs (e.g., alveolar wall thickening) (Stankus et al., 1978; Drew et al., 1974). Human relevant research on the acute inhalation toxicity of this aluminium salt is limited; therefore, we used A549 cells as an *in vitro* model of human alveolar cells to assess ACH's potential to induce oxidative stress, immunotoxicity, and epigenetic changes. A549 cells were exposed to three non-cytotoxic concentrations of ACH (0.25, 0.5 and 1 mg/mL) for 24 hours, and the flow cytometry-based tests were carried out. Our results showed that ACH induced the production of reactive oxygen species (ROS) (H2DCFDA probe) with a two-fold increase in median fluorescence intensity (MFI). However, no alterations on the released cytokine profile (Cytometric Bead Array (CBA) Human Inflammatory Cytokine kit) were observed, indicating that ACH may not induce inflammation on lung cells. Moreover, the global DNA methylation pattern (5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC)) and the presence of histone modification associated with DNA damage (phospho-histone H2AX (gamma-H2AX)) (immunostaining for flow cytometry) were not affected by ACH exposure. In conclusion, our data suggest that

ACH might be safe for the human respiratory tract concerning immunotoxicity and epigenetic changes, but it may induce oxidative stress on alveolar cells. Hence, further research is needed to ensure that the exposure to ACH via inhalation is safe.

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**Presentation:** Poster

1010

## Effective user support documentation for a toxicological data resource: ICE as a case study

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Access to high-quality *in vivo*, *in vitro*, and *in silico* data is essential to development and validation of new approaches for chemical safety testing. Sources of such data must address user requirements for accessibility, content, and usability. High-quality user documentation supports all three of these requirements, which are concordant with FAIR (Findable, Accessible, Interoperable, and Reusable) data principles. Improvement of user documentation is a key focus of each release of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) Integrated Chemical Environment (ICE). Since its launch in 2017, ICE has been continual-



ly expanded and improved in response to stakeholder feedback. Help resources provided by ICE include tooltips to explain features and terminology, dialogs that explain inputs and outputs in greater detail, metadata that provide context for download data, webpages within the ICE site that provide explanations of data sets and tools, and downloadable user guides for every ICE tool. During every ICE update, documentation is reviewed and improved using a process that leverages the expertise of subject matter experts and technical communicators to ensure accuracy and continuous improvement. This process has resulted in ICE having a body of user support documentation that has been recognized for its utility and quality by both stakeholders and an independent review of technical communications professionals. The presentation will describe the ICE user help resources and improvement process in detail, offering ICE as a case study in how effective documentation can be provided for a toxicological data resource.

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**Presentation:** Poster

1011

## Integrated approach to evaluate skin permeation and skin sensitization of *Baccharis trimera* (Less.) DC (Asteraceae)

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Skin sensitization is an important toxicological endpoint for cosmetic products, although one step still uses animals (OECD TG 429). In this context we are proposing the use of the concept of IATA – Integrated Approaches to Testing and Assessment combining the results of *in chemico* and *in silico* tests to provide a more realistic prediction when using alternative methods, in an attempt to reduce even more the substances that must be tested using the last step of the skin sensitization cascade. This work proposed a view on the capacity of skin permeation and covalent binding of four proficiency substances using *in silico* and *in chemico* approaches. The first one uses the software ADMET Predictor™ and QSAR Toolbox, in which the physicochemical properties of the substances were analyzed. *In chemico* tests were made following the DPRA protocol (OECD 442C) using 1-chloro-2,4-dinitrobenzene, butane-2,3-dione, cinnamaldehyde

and n-butanol as proficiency chemicals. The same procedures were also done with the extract of *Baccharis trimera*, which is a Brazilian plant rich in polyphenols with antioxidant and UV blocker activities with chlorogenic acid as his isolated marker. As the *in chemico* protocol is not validated for complex mixtures as plant extracts, this work also provides a light on this possibility, since it compares and discuss the potential of the extract as a sensitizer and its performance between different methodologies. The results show that is possible to combine the skin permeation parameters obtained with the *in silico* methods with the *in chemico* results, even when an isolated majoritarian substance is used as a substitutive for the complex mixture.

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**Presentation:** Poster

1013

## Dose-dependent cytotoxicity of bismuth nanoparticles produced by LASiS in a reference mammalian cell line BALB/C 3T3 and human mesenchymal stem cells

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Nanoparticles (NPs) have emerged as new potential tools for many applications in previous years. Among all types of NPs, bismuth NPs (BiNPs) have a very low cost and potential for many applications, ranging from medicine to industry. Although the toxic effects of bismuth have been studied, little is known about its toxicity at the nanoscale level. Therefore, in this study, we aimed to investigate the cytotoxic effects of BiNPs produced by laser ablation synthesis in solution (LASiS) in a reference mammalian cell line (BALB/c 3T3 cells) and human adipose-derived stem cells (ADSC). BiNPs were synthesized by LASiS and stabilized in two different solutions: culture medium supplemented with fetal bovine serum (FBS) and bovine serum albumin (BSA). Additionally, BiNPs were characterized by UV/Vis spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM) and zeta potential. The cytotoxicity of BiNPs



was evaluated through the neutral red uptake (NRU) and MTT assay. TEM was performed to evaluate the ultrastructural interaction between BiNPs and 3T3 cells and TUNEL assay was performed to assess the cell death mechanism. The cytotoxicity of BiNPs in BALB/c 3T3 cells through NRU using culture medium (IC<sub>50</sub>: 28.51 ± 9.96 µg/ml) and BSA (IC<sub>50</sub>: 25.54 ± 8.37 µg/ml) was assessed, and they were not significantly different. The LD<sub>50</sub> was predicted, and BiNPs were estimated as GHS class 4. We also found that cell death occurs due to apoptosis and ultrastructural level analysis suggest that BiNPs internalization originates myelin figures inside the cells. In preliminary assays, the cytotoxicity of BiNPs+BSA in ADSC through NRU (IC<sub>50</sub>: 271.35 µg/ml) and MTT (IC<sub>50</sub>: 243.08 µg/ml) was assessed, predicting BiNPs as GHS class 4 as well. To date, this is the first study to assess the cytotoxicity of BiNPs produced by LASiS and to predict the possible LD<sub>50</sub> and GHS class of BiNPs.

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**Presentation:** Poster

1015

### The inhibition of adipogenesis via an *in vitro* assay can reduce animal use by more precisely estimating the starting dose for the acute toxic class method

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We established an adipogenesis inhibition assay as an adequate and sensitive *in vitro* model for reducing animal use by estimating the starting dose for the acute toxic class (ATC) method. Human adipose-derived stem cells (ADSCs) underwent adipogenic differentiation induction for 14 days. Then, by high-content imaging analysis, we determined the percentage and area of cell differentiation that we considered suitable for negative and positive internal control according to the quality control criteria strictly standardized mean difference (SSMD) and robust SSMD. Moreover, we established sodium dodecyl sulfate (SDS) as an external positive control in this assay. To measure reduction in animal use to estimate the starting dose for the ATC method, we evaluated 10 chemicals representing Globally Harmonized System of Classification and La-

beling of Chemicals (GHS) toxicity categories 1-5 and unclassified toxicity and determined the dose-response curves for percentage and area of cell differentiation by using the Hill function with an R<sup>2</sup> ≥ 0.85. The resulting IC<sub>50</sub> values were used for LD<sub>50</sub> prediction and for estimating the starting dose for the ATC method. Our results indicated that use of the inhibition of adipogenesis assay to estimate the starting dose for the ATC method would decrease animal use for 7 out of 10 tested substances, possibly all substances if we consider the more toxic test substances in GHS categories 1, 2, and 3. We can conclude that the present assay is a suitable alternative to reduce animal testing in the first steps of predicting highly toxic substances. Moreover, this method also presents internal and external controls as differentials, which guarantee the quality of the assay as well as the results. These features are important for suggesting a methodology for regulatory purposes.

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**Presentation:** Poster

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### Development of 3D cultures of zebrafish liver and embryo cell lines by hanging drop and orbital shaking methods

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Acute fish bioassays are required to evaluate chemical toxicity in aquatic environments, and although the fish-embryo test has been adopted by OECD to refine the acute bioassay on adult fish, recent statistics warn of the high number of fish used. Fish cells have been proposed as an alternative to reduce the use of fish in ecotoxicology, and 3D fish cultures may be of great utility; however, the 3D culture methods need first to be well established for fish cells. For this study, we tested and compared the effectiveness (capacity of assembling fish spheroid, size uniformity, spheroidal shape) of different 3D culture methods (hanging drop (HD), orbital shaking (OS), HD combined with OS (HD+OS)) to generate spheroids



of zebrafish cell lines (ZFL – liver-derived, ZEM2S – embryonic-derived). Time in HD (3-5 days) and different rotational speeds (70 and 100 rpm) in flat- or round-bottom 96-well plates in OS were evaluated. HD was not able to form ZFL spheroids, whereas HD (5 days) + OS (5 days) successfully formed ZFL spheroids. The OS method formed ZFL spheroids only in the round-bottom plate, and 70 rpm was the best rotational speed tested. ZFL spheroids of both HD+OS and OS (70 rpm) were reproducible in size ( $177.50 \pm 2.81$  and  $225.62 \pm 19.20$   $\mu\text{m}$ , respectively) and circularity ( $0.83 \pm 0.02$  and  $0.80 \pm 0.01$ , respectively). The OS method in round-bottom plate also formed reproducible ZEM2S spheroids in 1 day ( $226.23 \pm 0.57$   $\mu\text{m}$  diameter and  $0.80 \pm 0.01$  circularity). OS method was the fastest (5 days) and simplest 3D spheroid method, while HD + OS required considerable time (10 days) to fully form ZFL spheroids, and it is labor-intensive. This study contributes to identify a fast, reproducible, and simple protocol to generate single piscine spheroids in 96-well plates and supports the application of fish 3D models in the industry and academic laboratories.

**Presentation:** Poster

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## Pectin-based 3D printed models for nervous tissue

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**Introduction:** Two-dimensional cultures are limited in modeling the complexity of chemical and physical stimuli that characterize the nervous tissue (Zhuang et al., 2018). To fabricate hydrogel-based multi-layered structures with accurate control over the arrangement of materials and cells, recent advances have focused on 3D printing (Knowlton et al., 2018). In this work, we exploited pectin, a versatile class of anionic and branched polysaccharides to develop bioinspired, extrusion-based bioinks as suitable candidates for *in vitro* models of nervous tissue.

**Materials and Methods:** Pectin was dissolved in 0,9% w/v NaCl or DMEM and hydrogels were prepared by internal gelation with calcium carbonate (Secchi et al., 2014). For both solutions and hydrogels, viscoelastic properties were characterized by rheological measurements at 0, 30 and 60 minutes after mixing. For DMEM preparations, indirect cytocompatibility was assessed with SH-SY5Y neuroblast-like and C8D1A astrocyte cells by MTS assay up to 7 days. After printing (INKREDIBLE+, CEL-LINK), filament width dependence on time, printing velocity, nozzle diameter and pressure were studied.

**Results:** All the produced hydrogels showed shear-thinning and self-healing properties, with an elastic modulus compati-

ble with brain tissue (Axpe et al., 2020). For all the tested time points, hydrogel supernatants did not reduce SH-SY5Y and C8D1A cell metabolic activity with respect to standard medium. At 0, 30 and 60 minutes, hydrogels formed continuous filaments with over 85% shape fidelity. The width of the printed fibers was directly proportional to printing pressure. At 0 and 30 minutes, both preparations were extruded at pressures under 45 kPa, compatible with cell survival (Fantini et al., 2019).

**Conclusions:** Pectin-based 3D printed hydrogels were developed to reproduce the structural organization of the nervous tissue.

**Acknowledgements:** This work has been supported by Fondazione Cariplo, grant n° 2019-4615.

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**Presentation:** Poster

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## Transitioning A549 cells to FBS-free media: Process and determining success

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Replacing the use of fetal bovine serum (FBS) in cell culture media bolsters the reproducibility of *in vitro* research and overcomes the ethical and legal challenges associated with its use. Increasingly, scientists are focusing on replacing the use of FBS as a supplement in cell culture media with chemically defined, xeno-free, or serum-free components.



Here we describe replacements for FBS, including their advantages and disadvantages. Using A549 cells, an immortalized human epithelial alveolar cell line commonly used in respiratory research, as a case study, we demonstrate the process of transitioning cell culture medium containing FBS to four types of commercially available media containing only chemically defined, xeno-free, or serum-free components and for cryopreservation of transitioned cells. To determine whether the transition was successful, numerous techniques were used to characterize the cells. 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); monitoring the expression of relevant genes; and determining surfactant production (surfactant droplet test). 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 cells. This case study has the potential to be used as a template to guide the transition and evaluation process for replacing FBS for other cell lines.

**Presentation:** Poster

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## Application of meta-analysis to derivation of points-of-departure for short-term inhalation exposure levels of hazardous chemicals

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For short-term chemical inhalation exposures to hazardous chemicals, the incidence of a health effect usually conforms to a generalized linear model with a bivariate probit link function dependent on inhalant concentration (C) and the duration of exposure (t). The National Academy's Acute Exposure Guideline Levels (AEGLs) Committee relies on this model when deriving AEGLs. Threshold concentrations at AEGL durations are established by the toxic load equation,  $C^n \times t = \text{constant}$ , which Toxic Load Exponent n (TLE or n-value) directly follows from the probit model. When multiple probit datasets are available, often they cannot be directly pooled together because of disparities in experimental design. Recently, we reported both a meta-analytical framework for multiple studies (Prussia et al., 2020) and an application of the new methodology to dimethyl sulfide (Demchuk et al., 2018). The new methodology allows accurate TLE estimation even if multiple datasets are heterogeneous. The utilized techniques include categorical regression, common-effect and random-effects models. The proposed framework was applied to multiple-study datasets from AEGL technical support documents.

Using the framework, both new TLEs and confidence intervals on them were derived. The recalculated TLEs and points-of-departure include ammonia (n = 2.13, 95% CI: 1.98-2.27), allyl alcohol (n = 0.95, 95% CI: 0.76-1.15), 1,1,1-trichloroethane (n = 3.46, 95% CI: 2.85-4.07), carbon tetrachloride (n = 2.51, 95% CI: 2.27-2.76), and oxygen difluoride (n = 1.29, 95% CI: 0.26-2.32). Next, the new TLEs and their confidence intervals were applied in calculations of short-term inhalation points of departure at five reference AEGL durations.

*Disclaimer: The findings and conclusions in this presentation have not been formally disseminated by the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry and should not be construed to represent any agency determination or policy.*

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**Presentation:** Poster

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

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Animal-derived components, including fetal bovine serum, coating materials, growth factors, dissociation enzymes, and antibodies are routinely used in cell/tissue culture and other *in vitro* laboratory practices. In addition to ethical issues, the use and production of animal-derived materials and reagents raises many scientific concerns regarding experimental reproducibility and biosafety (OECD, 2018; van der Valk et al., 2018; Pinheiro de Oliveira et al., 2013; Gray et al., 2016). To map the current use of these reagents across multiple sectors and to identify the obstacles



preventing the implementation of non-animal derived alternatives, we have launched a global online survey aimed at understanding: 1. What are the animal-derived materials and reagents most commonly used in *in vitro* experimentation, 2. What are the main issues perceived as associated with the production and use of animal-derived materials and reagents, 3. What is the current level of knowledge on available non-animal derived alternative materials and reagents and 4. What educational and information sources could be useful or most impactful to disseminate knowledge on these non-animal derived alternative materials and reagents. This survey caters to any professionals active in the human health, animal or life science area at any level or representatives of any life science-related institution. Survey results will be used for creating a framework to shape the policy in science and education. The ultimate goal is to promote a fully replacement of animal-derived materials to ensure data reproducibility and reliability, as advocated in the Guidance Document on Good In Vitro Method Practices (GIVIMP), and in accordance with EU Directive 2010/63.

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**Presentation:** Poster

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## Adopting EU research category/ subcategory nomenclature to categorize research uses of dogs

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As part of our project to examine the use of dogs in invasive biomedical research in US laboratories, various types of data were collected from publications and grants. The first category of da-

ta collected was “research type,” and a simple nomenclature was needed for this application. Existing nomenclatures, controlled vocabularies, and ontologies, including Medical Subjects Headings (MeSH) (NLM, n.d.), were examined and found too lengthy and/or complex, or otherwise inadequate. However, the research categories and subcategories established by the European Union (EU) to categorize animal research for EU statistical reporting requirements (EUR-Lex, 2012) did provide useful terminology for this application.

One of three research categories and a subcategory were identified for each publication and grant as follows: (1) “basic research” with 13 subcategories, (2) “translational/applied research” with 25 subcategories, or (3) “regulatory use.” Several EU subcategories were not used. A secondary subcategory was added to further categorize “basic research/non-regulatory toxicology” and “regulatory use.”

The significance of reporting this use of the EU nomenclature is two-fold. First, in contrast to the EU, the United States Department of Agriculture (USDA) and other US institutions do not collect detailed animal use statistics, and therefore do not have a similar nomenclature of animal research categories. Adopting an established and internationally recognized nomenclature, along with requiring detailed animal use reporting, would benefit the many researchers and agencies needing this kind of data. Second, having a simple and established nomenclature to categorize animal use in publications and grants would benefit researchers internationally who need this information to address 3Rs goals.

The retroactive identification of research categories for publications and grants in this study was time consuming and difficult, with potential errors due to insufficient information. Therefore, we recommend animal use category assignments be provided by researchers as part of grant or manuscript submission.

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**Presentation:** Poster



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## Evaluation of cellular damage using *in vitro* intestinal models after exposure to tetrabromobisphenol A and polystyrene nanoplastics

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Nanoplastics (NPs), small particles with sizes from 0.1 to 0.001  $\mu\text{m}$ , tend to accumulate in the ecosystems, causing negative effects on the environment. Potential implications in human health of these emerging pollutants of global concern that can also be carriers of other potentially toxic chemicals remain largely unknown. The aim of the present study was to evaluate whether a co-exposure of the brominated flame retardant tetrabromobisphenol A (TBBPA) and polystyrene nanoparticles (NPs) might potentiate the individual toxic effects of these compounds in human intestinal cells. In the first set of experiments, we assessed the acute (24 h) basal cytotoxicity of both compounds (TBBPA 1-150  $\mu\text{M}$ , 0.544-81.6  $\mu\text{g/mL}$ ; NPs 0.1-200  $\mu\text{g/mL}$ ) on Caco-2 cells and Caco-2/HT29-MTX co-culture model, using three complementary parameters (metabolic activity, plasma membrane integrity, and lysosomal integrity). The most relevant conditions were selected for chronic exposure for 21 days, encompassing the differentiation period of these cells. We also tested the genotoxic effects using the alkaline version of the Comet assay. With the co-culture model, we observed at the selected combined concentrations (10  $\mu\text{M}$  TBBPA + 50  $\mu\text{g/mL}$  of NPs) a significant reduction in metabolic activity after 21 days according to the Alamar Blue assay, although no effects were detected with those same conditions for the individual exposures. Interestingly, the integrity of the plasma and lysosomal membranes remained unchanged with these conditions. In addition, preliminary results suggest the induction of DNA damage after 24 h combined treatments. Thereby, our results suggest a potential interaction between the two tested compounds, revealing that the polystyrene nanoplastics are able to increase the toxic effects produced by TBBPA in intestinal human cells.

**Presentation:** Poster

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## Towards a novel alternative toxicity model with normothermally perfused *ex vivo* livers based on whole porcine slaughterhouse organs

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The development of highly predictive liver models for toxicity screenings is both important and challenging. *Ex vivo* machine perfusion has emerged in the last years as a promising tool to maintain organ functions and complexity. Research in this field is focusing primarily on organ transplantation and extracorporeal support rather than on long-term *ex vivo* perfused organ platforms for pre-clinical research purposes. We here present a normothermic *ex vivo* liver perfusion model based on slaughterhouse material for interventional studies to test new medical devices or to study acute tissue response to drug treatment.

After procurement of the liver at the slaughterhouse, the liver is attached to an extracorporeal perfusion circuit and re-perfused at 39°C. Pressures of 10 and 80 mmHg are applied to veins and arteries, respectively. Flows reach physiological levels owing to resistance of the organ itself. To assess liver function, we performed the indocyanine green (ICG) test, blood gas analysis, quantified bile production and oxygen consumption.

In our study, a simplified normothermic perfusion model was developed to successfully perfuse an *ex vivo* liver obtained from a slaughterhouse. Functionality and viability were demonstrated by continuous bile production (Grosse-Siestrup et al., 2001), which was confirmed by an ICGt1/2 result comparable to literature (Schreiter et al., 2016) and stable oxygen consumption.

The platform allows research on biological pathways or biomechanics of the liver, and it is an ethically well-considered partial alternative in pre-clinical studies to reduce animal testing. Further development and circuit optimization will lead to a long-term liver model and a novel versatile platform for interventional studies and drug treatment.

*Acknowledgment: The project was funded by EU's Horizon 2020 research program under the Marie Skłodowska-Curie grant agreement No.860715.*

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**Presentation:** Poster



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## Exploiting the use of iPSC derived renal proximal tubular like cells to investigate megalin mediated aminoglycoside toxicity

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Exposure to environmental pollution and certain pharmaceuticals can adversely affect the kidney and contribute to the acceleration of chronic renal disease which is a heavy burden on health care systems worldwide. While human renal cell lines, such as the RPTEC/TERT1 cells are useful tools to study nephrotoxicity, they represent only one genetic background and may have acquired and lost some typical characterizations in culture. One such example is the absence of megalin (LRP2) expression in most of the existing 2D *in vitro* proximal tubular models. iPSC derived models are promising new tools that may be able to overcome some of these limitations. This study investigates the utility of a newly developed iPSC protocol for deriving proximal tubule-like cells (PTL) and the ability of this model to study megalin-mediated aminoglycoside toxicity. Undifferentiated iPSC were differentiated into PTL and temporal alterations of the cells were characterized by assessing the expression of pluripotency markers, renal developmental markers, and maturation markers via immunofluorescence and western blot analysis. Differentiated PTL cells exhibited a polarized phenotype, barrier formation, and expression of the functionally active proximal tubule-specific marker, megalin. To compare the effects of megalin-facilitated aminoglycosides uptake on different proximal tubular models, iPSC derived PTL, human primary proximal tubular cells, RPTEC/TERT1, and HK2 were exposed to aminoglycosides (gentamicin and tobramycin) at the concentration of 12, 250, and 450 µg/ml. Transcriptomic alterations were quantified using the TempO-Seq assay (BioClavis, Ltd). iPSC derived PTL and primary proximal tubular cells showed similar responses with increased interferon signaling and anti-viral response in both gentamicin and tobramycin exposure. On the other hand, RPTEC/TERT1 and HK2 cells didn't show any predominant pathway activation. In conclusion, iPSC derived PTL represents an improved human-relevant model for nephrotoxicity and could provide an opportunity to study the effects of compounds that are taken up through megalin-facilitated endocytosis.

**Presentation:** Poster

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## 3Rs info hub

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The 3Rs info hub bundles current and relevant 3Rs multimedia content into a single information portal.

A critical selection of the 3Rs-related multimedia content available on the internet has been made in order to present relevant material in one hub in a structured way. Animations, presentations, online-seminars, podcasts and video contributions addressing 3Rs were categorized, e.g., by level of prior knowledge required, 3R method and organ system addressed. Assigning keywords were given to each content and make it easy for the users to find the topic they are looking for. Materials can easily be accessed via links from the 3Rs info hub rather than being overwhelmed by the plethora of search engine results and losing track of the numerous studies and procedures available.

We started this project with pharmacologically and toxicologically relevant organ systems, but the hub will be extended to other organ systems soon. The site was deliberately designed in English to allow worldwide use.

In addition to the multimedia content links, learning scenarios were developed for each topic addressed. Face-to-face teaching can frame the use of the 3Rs info hub: While the e-learning elements serve the purpose of knowledge acquisition in self-study, classroom teaching consolidates, applies and discusses what has been learned. With the 3Rs info hub, lecturers have a preselected content and material to design a seminar according to their needs.

Each module contains a learning success control (3Rs quiz). This allows students to check what they have learned in a fun way without jeopardizing their grade point average.

In order to involve students directly in the development process and to implement the research oriented 3Rs teaching, we have created a podcast series called StudentCast on 3Rs. In the episodes students discuss with experts common 3Rs topics and current research on non-animal methods.

**Presentation:** Poster



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## Effect of gnawing wood enrichment on disease induction in a mouse model for diet-induced NASH

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Non-alcoholic steatohepatitis (NASH) is the most prevalent form of chronic liver disease worldwide. Currently no approved pharmacological treatment is available for use in patients. Mouse models which rely on diet-induced development of NASH are used in the preclinical drug development phase for novel therapeutics. Gnawing wood sticks commonly serve as cage enrichment in mouse studies but can potentially affect intestinal uptake and/or result in faecal excretion of dietary components following binding or attachment to wood fibres. In the current study we investigated whether addition of gnawing wood cage enrichment could affect the dietary induction of NASH in mice.

APOE\*3Leiden.CETP mice, a translational animal model that displays histopathological characteristics of NASH in the context of obesity, insulin resistance and hyperlipidemia were used in this study. To induce NASH, mice were fed a high fat and cholesterol (HFC) diet for 25 weeks (HFC control group) with (n = 15) or without (n = 15) addition of gnawing wood as cage enrichment. The effects on body weight, food intake, plasma parameters and NASH histopathology were assessed.

Addition of gnawing wood led to a similar obese body weight in the mice, nor did it affect food intake. Blood glucose levels were similar between both groups and in both groups hypercholesterolemia and hypertriglyceridemia were induced by the diet to a similar extent. Liver weights were similar between both groups and histopathological evaluation revealed that macrovesicular steatosis, microvesicular steatosis, hepatic inflammation and hepatic fibrosis were all similarly induced as well.

Addition of gnawing wood did not affect the induction of NASH and fibrosis in HFC fed APOE\*3Leiden.CETP mice and can be used as cage enrichment in this type of diet-induced disease model.

**Presentation:** Poster

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## Associations between clinical signs and pathological findings in toxicity testing

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Animal testing for toxicity assessment of chemicals and pharmaceuticals must take the 3R principles into consideration. During toxicity testing *in vivo*, clinical signs are used to monitor animal welfare and to inform about potential toxicity. This study investigated possible associations between clinical signs, body weight change and histopathological findings observed after necropsy. We hypothesized that clinical signs and body weight loss observed during experiments could be used as early markers of organ toxicity. This represents a potential for Refinement in terms of improved study management and decreasing of pain and distress experienced during animal experiments. To this end, data from three sequential toxicity studies of an anti-cancer drug candidate in rats were analyzed using the multivariate partial least squares (PLS) regression method. Associations with a predictive value over 80% were found between the occurrence of mild to severe clinical signs and histopathological findings in the thymus, testes, epididymides and bone marrow. Piloerection, eyes half shut and slightly decreased motor activity were most strongly associated with the pathological findings. A 5% body weight loss was found to be a strong empirical predictor of pathological findings but could also be predicted accurately by clinical signs. Thus, we suggest using mild clinical signs and 5% body weight loss as toxicity markers and as a non-invasive surveillance tool to monitor research animal welfare in toxicity testing. These clinical signs may also enable Reduction of animal use due to their informative potential to support scientific decisions regarding drug candidate selection, dose setting, study design, and toxicity assessment.

**Presentation:** Poster

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## Extension of the performance statistics of defined approaches to distinguish between the three UN GHS categories for eye hazard identification and beyond

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In recent decades, considerable progress has been made in the field of alternatives to animal testing to assess the safety of chemicals. In order to determine the performance of these methods, the results are compared with reference data (animal or human) based on a confusion matrix, a cross table that reports the predicted classes against the reference classes. In case of a binary response, sensitivity and specificity are widely used statistical measures of performance. In case of a multi-class response (three or more categories), sensitivity and specificity can be calculated based on a one-vs-all approach and provide information on the class-specific performance.

Several *in vitro* test methods have been OECD-adopted to identify chemicals causing serious eye damage (Cat.1), or to identify chemicals not requiring classification and labelling for eye irritation or serious eye damage (No Cat.). Their predictive capacities (accuracy, sensitivity, false negatives, specificity, and false positives) are generally computed based on a 2x2 confusion matrix. Recently, Cosmetics Europe developed two defined approaches (DAs) for eye hazard identification that distinguish between the three UN GHS categories, including Cat. 2 defined as chemicals causing reversible effects on the eye (Alépée et al., 2019a,b). The overall performance of the DAs was assessed based on 3x3 confusion matrices. To evaluate the class-specific performance, the 3x3 matrix is converted into three 2x2 matrices and sensitivity, specificity, and balanced accuracy are calculated for each matrix separately. This is the first time that this approach is applied to eye hazard identification considering the three UN GHS categories. While the focus is on the performance of alternatives methods/defined approaches for eye hazard identification, the computations shown here with a numerical example are also applicable to other domains of hazard identification and to more than 3 categories.

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neat liquids based on Cosmetics Europe Analysis of *in vitro* RhCE and BCOP test methods. *Toxicol In Vitro* 59, 100-114. doi:10.1016/j.tiv.2019.04.011

Alépée, N., Adriaens, E., Abo, T. et al. (2019b). Development of a defined approach for eye irritation or serious eye damage for liquids, neat and in dilution, based on cosmetics Europe analysis of *in vitro* STE and BCOP test methods. *Toxicol In Vitro* 57, 154-163. doi:10.1016/j.tiv.2019.02.019

**Presentation:** Poster

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## Is it possible to stem the tide of GA animal use in biomedical research?

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The use of genetically altered (GA) animal models has grown significantly in recent years with GA animals now being used in 42% of all experimental procedures in the UK. This percentage has doubled in the past decade (Home Office, 2020). The breeding and maintenance of GA animal lines alone accounts for 1.67 million procedures. The ability to manipulate mouse genes to enable the simulation of aspects of human diseases such as diabetes, motor neuron disease or others, is considered cutting edge science and vast amounts of time, money and energy are dedicated to improving the methodology. The advent of CRISPR has allowed genetic modification to become easier and more accessible, whilst a growing body of published literature in this field fuels further investment and demand for funding.

However, given that all research funding has an opportunity cost, could or should this time and funding be more appropriately directed to more relevant human-based methods, and what would be required to make this shift? There are significant and unavoidable problems with using GA models, most notably that the human diseases under investigation often have significant lifestyle and aging factors which can't be replicated. In addition, GA lines are understandably closely controlled to ensure that there is little to no natural variability in phenotypes. Once again, this fails to adequately replicate the behaviour of a disease within the human population. Finally, there is a significant welfare concern around the suffering of these animals and whether this is warranted. FRAME is commissioning a piece of research to understand the motivations of leading GA animal researchers for using animal models over alternatives, investigate the perceived strengths and weaknesses of these models in different areas of research, and identify the current gaps that prevent researchers using non-animal methods.

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**Presentation:** Poster

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## Gene network regulations show different signatures for morpholines and piperidines in the ESTc

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One of the *in vitro* embryotoxicity screens is the cardiac embryonic stem cell test (ESTc), which uses cardiomyocyte formation as the main differentiation route. Studies are ongoing into whether a wider use of the biological domain may contribute in broadening the applicability of the test, to prioritize structurally similar chemicals for its safety. The use of gene transcript biomarkers would contribute to a quantitative readout of the test. But which biomarkers could be useful for safety predictions? To answer this question, the morpholines (tridemorph – TDM; fenpropimorph – FPM) and piperidines (fenpropidin – FPD; spiroxamine – SPX) were tested. These compounds cause fetal malformations in the rat such as cleft palate. The formation of cleft palate in general is linked to interference with retinoic acid balance, neural crest cell migration, and cholesterol biosynthesis. Markers related to these pathways were tested at low (ID10) and high (ID50) concentrations after 24 hours and 7 days of exposure. All tested compounds showed stimulating effects on the cholesterol biosynthesis related marker *Msmo1* after 24 h exposure and TDM showed *Cyp26a1* inhibition which codes for an enzyme that inactivates retinoic acid. A long exposure duration stimulated expression levels for pluripotency (*Pou5f1*), but also for the ectodermal (*Nes*) and endodermal (*Gata4*) germ layer markers. Differentiation markers were affected for cardiomyocyte (*Nkx2-5*; *Myh6*), neural (*Tubb3*), and neural crest cell development (*Ap2α*; *Msx2*; *Snai2*). These gene expression levels were captured in gene-networks which showed clear differences in signatures between the morpholines and piperidines, especially at low concentrations after 7 days of exposure. The different biomarker response profiles gave more mechanistic insight into the compound interferences

in the tested differentiation routes which could be incorporated into the safety predictions.

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## Performance statistics of defined approaches for eye hazard identification of liquids to distinguish between the three UN GHS categories

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Cosmetics Europe develop two defined approaches (DAs) for eye hazard identification, i.e., addressing both serious eye damage and eye irritation (or the absence thereof), for non-surfactant liquid test substances. DAL-1 combines four physicochemical properties, the Reconstructed human Cornea-like Epithelium (RhCE: EpiOcular™ EIT (VRM1) and SkinEthic™ HCE EIT (VRM2)) test method (OECD TG 492), and Bovine Corneal Opacity and Permeability (BCOP) test method (OECD TG 437). DAL-2 combines the Short Time Exposure (STE) test method (OECD TG 491) and BCOP test method (OECD TG 437). In both DAs, the BCOP laser light-based opacitometer (LLBO) is used, as described within the OECD TG 437, however only the opacity is used to identify liquids that cause serious eye damage. The performance of the DAs to distinguish between the three UN GHS categories were compared against the minimum performance values for each category proposed by the OECD Expert Group on Eye/Skin Irritation/Corrosion and Phototoxicity.

Note that 16 Cat. 1, 21 Cat. 2, and 31 No Cat. reference chemicals were tested in common by the DAs. The balanced accuracy (average of the % correct predictions of each category) of DAL-1 with VRM1, DAL-1 with VRM2, and DAL-2 was 69.2%, 75.2%, and 74.3%, respectively. DAL-1 with VRM1 identified 76.5% of Cat. 1 (N = 17), 59.1% of Cat. 2 (N = 22) and 72.1% of No Cat. (N = 55) correctly (69.2). DAL-1 with VRM2 identified 76.5% of Cat. 1 (N = 17), 68.7% of Cat. 2 (N = 23) and 80.4% of No Cat. (N = 46) correctly. DAL-2 identified 81.2% of Cat. 1 (N = 17), 56.3% of Cat. 2 (N = 24) and 85.3% of No Cat. (N = 123) correctly. These values were greater than the proposed minimum



performance of 75% for Cat. 1, 50% for Cat. 2 and 70% for No Cat. Furthermore, the class-specific performance metrics are also provided for each DA.

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**Presentation:** Poster

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## ECM-based *in vitro* 3D-models of the liver for hepatotoxicity testing

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In the context of drug development, liver plays a crucial role. It is not only directly responsible for drug metabolism, but it is also implied in hundreds of viable functions, whose impairment might produce differences in active principle processing, and pharmacokinetics, leading to the outbreak of severe adverse reactions, including hepatotoxic effects. This work will present the first developmental steps of an ECM-based, *in vitro* 3D-model of the liver, to assess drugs hepatotoxicity. ECM was obtained from decellularized porcine liver, by a combination of different methods (Mazza et al., 2017). The decellularization buffer was injected in multiple sites of 0.5 cm cubes of liver and then used to incubate the samples while under orbital stirring up to 7 days. Lyophilized cubes were grinded after freezing in liquid N<sub>2</sub>. ECM powder (1.4% w/v) was added in an alginate (ALG) solution (3.5% w/v) in complete medium. The hydrogel was characterized by rheological testing and the stability in medium was evaluated up to 14 days. For cell loaded hydrogels, HepG2 cells were suspended in the ALG-ECM suspension (2x10<sup>6</sup> cell/ml)

prior crosslinking (Lan et al., 2010). MTT test and confocal microscopy, with live/dead kit were employed to evaluate viability and spatial distribution. The produced hydrogel shows rheological characteristics reproducing the ones of the liver tissue and is stable up to 11 days. Loaded HepG2 cells were viable and homogeneously distributed within the 3D-matrix. Their number increased upon time and the production of physiological-like aggregates is observed. The model is now being employed for the study of hepatotoxicity to the administration of various drugs (i.e., acetaminophen, midazolam, chlordiazepoxide). Furthermore, the developed model can be tailored to mimic not only physiological organs, but also diseased ones; this study is integrated in the development of new approach methodologies applied to toxicology and preclinical drug evaluation.

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**Presentation:** Poster

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## Implementation of an *in vitro* transgenic rodent (TGR) assay for the detection of potential mutagens and assessment of their mechanism of action

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The TGR assay (OECD 488) is an *in vivo* test addressing gene mutations. Hereby, the bacterial lacZ gene, amongst others, is used as a reporter gene to detect mutations easily and reliably. Multiple copies of this gene are integrated in the mouse chromosome. An *in vitro* version of this mutagenicity assay has been described by Cox et al. (2019). In this *in vitro* version, “*in vitro* TGR assay”, primary hepatocytes (PHs) isolated from transgenic mice (MutaMouse) instead of the living animal are treated with the test substance. Due to the similarity of the *in vivo* and the corresponding *in vitro* assay, this approach deems to predict the *in vivo* outcome better than other *in vitro* genotoxicity assays.



This *in vitro* test system was validated in our laboratory by using 6 different gene mutagens each bearing its own characteristic mechanism of action and requirement for metabolization. All tested mutagens, except cyproterone acetate, showed an increase in lacZ MF, ranging from 4- to 13-fold compared to vehicle control.

Thus, the *in vitro* TGR assay was able to detect the mutagenic potential of direct-acting and pro-mutagens with a performance qualitatively similar to the *in vivo* TGR assay. The current investigations aim at sequencing the transgene in order to obtain a mutagenic fingerprint for the respective mechanism of mutagenic action.

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**Presentation:** Poster

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## Assessing human carcinogenicity risk without the rodent cancer bioassay

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The rodent cancer bioassay is often conducted when it may not be needed for human health risk assessment. As part of a collaborative effort, the Rethinking Carcinogenicity Assessment for Agrochemicals Project developed a reporting framework to guide a WoE evaluation for rodent cancer bioassays that can be waived as a data requirement. The framework is the result of an iterative process of writing waivers, regulatory agencies' reviews, and framework revision. The example waivers used to develop the framework were written for registered pesticide active ingredients in which the necessary data and information could be obtained through risk assessment documents or data evaluation records from the US EPA. This exercise was critical to the development of a draft framework, but it lacked authenticity; the regulators reviewing the waiver already knew the outcome of the rodent cancer bioassay(s). Syngenta has expanded the evaluation of the ReCAAP reporting framework by writing three case studies for new AIs where the data packages have not yet been submitted for registration. Waivers followed the established frame-

work considering ADME, potential exposure, sub-chronic toxicity, genotoxicity, immunosuppression, hormone perturbation, MOA and risk assessment of each, using a WoE evaluation. A thorough read across assessment was conducted to compare data on registered chemicals that were of a similar pesticidal MOA or shared structural similarity to support the prediction of chronic toxicity and/or tumorigenic potential. The case studies represent a range of different scenarios, from a new molecule in a well-established chemical class with a known MOA to a molecule with a new pesticidal MOA and limited read across to related molecules. Key learning from the case studies along with feedback from regulatory agencies will be presented. This effort represents an important step to establish criteria for waiving the rodent cancer bioassay(s) while ensuring a health protective chronic risk assessment.

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## Weight of evidence approach for skin sensitization potency categorization of fragrance ingredients

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Reliable human potency data is necessary for appropriate evaluation of new non-animal methods for skin sensitization potency prediction. The potency information is also critical for the quantitative risk assessment. Previously, human skin sensitization potency of fragrance materials was derived primarily using human data (Api et al., 2017). However, human testing is not typically conducted to explore the dose-response of a chemical. Rather, the human testing is conducted at a single dose that is expected to be safe. For this reason, the No Observed Effect Level (NOEL) from the human test can be close to, or well below, the threshold of the induction of sensitization. This means that human data alone may not correlate well with the actual potency of a given material. Therefore, we propose a Weight of Evidence (WoE) approach for potency categorization, where all existing data should be considered collectively. To demonstrate this approach, human, animal, *in vitro*, *in chemico*, and *in silico* data on 106 fragrance materials were evaluated to set skin sensitization potency categories (Extreme, Strong, Moderate, Weak, Very Weak and Non-Sensitizer). None was considered an extreme sensitizer, while 6, 23, 41, and 28 materials were categorized as strong, moderate, weak, and very weak sensitizers, respectively. Eight materials lacked evidence for the induction of skin sensitization. The skin sensitization potency categories for fragrance materi-

als based on the WoE approach are provided in this work. The WoE categories can serve as references for evaluation of the new non-animal methods, as well as for the quantitative risk assessment.

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**Presentation:** Poster

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## Effect of mineral fibers on acute toxicity and inflammation in an *in vitro* model of human M0-M1-M2 macrophages

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Inhalation of mineral fibers leads to chronic lung inflammation and the subsequent development of lung malignancies. Macrophages have a fundamental role in both triggering and maintaining the inflammatory response in the lung tissue. It is also known that cell damage is a necessary step for triggering the inflammatory response by immune cells. Three types of differentiated macrophages can contribute to the chronicization of the inflammatory response in the lung, namely M0, M1 and M2 cells corresponding to non-activated, pro-inflammatory and alternatively activated macrophages. Since until now, no comparative data are available on the acute toxicity and inflammatory response of M0, M1 and M2 macrophages challenged with mineral fibers, we set up an *in vitro* model based on human THP-1 monocytes, to study the acute effects of three types of toxic/carcinogenic mineral fibers, namely crocidolite, chrysotile and erionite.

After differentiation, the three macrophage phenotypes obtained were exposed to fibers for 24h. Cytotoxicity was quantified by the MTT test, LDH release, ROS production and apoptosis induction. DNA damage was investigated by gamma-H2AX foci detection and inflammation was studied by cytokine release quantification (IL-1beta and MCP-1). The results indicate different effects

of the fibers towards the three macrophage phenotypes. Different rates of cell toxicity, apoptosis, ROS production, DNA damage and cytokine release were observed for the three macrophage phenotypes with the fibers, with M0 and M1 macrophages showing higher rates of responses than M2. In general, at 24 h, crocidolite and chrysotile were more potent with respect to erionite in triggering apoptosis and cytokine release, while erionite had the highest effect in intracellular ROS production and, apparently, DNA damage. Overall, this *in vitro* model allows to perform accurate toxicity and inflammation comparative studies on the effects of mineral fibers on the three macrophage phenotypes, which could allow more accurate previsions of their carcinogenic effect.

**Presentation:** Poster

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## An alternative *in vitro* approach to evaluate in human endothelial cells and monocytes both direct and indirect carcinogenic effect of asbestos fibers

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Among mineral fibers, asbestos (i.e., chrysotile and crocidolite) and fibrous erionite are classified as carcinogens (Sayan et al., 2016). Two mechanisms of cyto-genotoxicity are proposed: (1) physical interference with the cellular processes, (2) stimulation of oxidative imbalance by increasing reactive oxygen species (ROS) production (Srivastava et al., 2010).

In order to investigate them, in this study a human endothelial (HECV) and a monocyte-like THP-1 cells were exposed, directly (dc) or indirectly (ic), to crocidolite, chrysotile and erionite. Briefly, 50 µg/mL of dissolved fibers were added to culture medium in dc-experiments, while in ic-assays the same amount was placed into Transwell® PET inserts (0.4 µm pore size).

Fiber-induced effects were investigated in terms of proliferation index (PI, DNA content assay; Rao et al., 1992), ROS production (dichlorofluorescein assay), DNA oxidative damage and inflammatory response.



In THP-1, 48 h of dc-treatment reduced PI by 20%, while, surprisingly, in ic-exposure crocidolite and erionite increased PI by 10 and 20%, after 48 h and 72 h, respectively.

On ic-THP-1, 4h of crocidolite, chrysotile and erionite treatments increased ROS production by 50, 90, and 150%, respectively, while in dc-HECV only chrysotile slightly stimulated ROS production (~20%). In both cell lines ROS over-production was accompanied, at 24 h, by the phosphorylation of H2AX, a marker of DNA damage.

On dc-HECV, chrysotile and erionite increased proinflammatory IL-1 $\beta$  cytokine and MCP-1 chemokine release (ELISA), after 24 h and 72 h, respectively. On dc-THP-1 all experimental treatments increased IL-1 $\beta$  and MCP-1 levels, suggesting a may-or susceptibility to activation of cellular damage-response. In ic-THP-1 these markers were slightly secreted in the medium but increased their intracellular content (10 folds vs basal levels).

These findings, although preliminary, suggest that in HECV and THP-1 the oxidative unbalance is responsible for both cytotoxic and pro-inflammatory action of the fibers.

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**Presentation:** Poster

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### Impedance spectroscopy as tool to determine concentration-dependent eye irritation effects

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Ocular irritancy testing on animals for safety reasons such as the Draize eye test are still prevalent around the globe. The heightened sensitivity of the eye requires a particularly strict control of chemicals. In the Global Harmonized System (GHS), neat chemicals are categorized 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 analyze reconstructed cornea like models based on primary human cells. By using impedance spectroscopy cell barrier properties are detectable via adjusted frequency ranges. We tested four category 1 substances in three different dilutions: 100%, 5% and 1%. Tracking concentration dependent damage, we measured impedance six times over 11 days. The transepithelial electrical resistance at the frequency of 1000 Hz (TEER1000Hz) 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.

Summarized, impedance spectroscopy indicated substance-related irritation effects of diluted testing substances in our cornea like model. Impedance-based predictions could influence the labelling of formulated consumer goods and pave the way to alternative tests.

**Presentation:** Poster

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### The development of an ex vivo organ model to study prolonged viability of individual organs and evaluate the perinatal life support system prototype

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Every year, 800,000 babies are born extremely preterm (EP; < 28 weeks of age) worldwide. A large proportion of survivors face lifelong disabilities, including breathing, cardiac, neurological, and metabolic problems. Current treatment requires the preterm initiation of body functions for which the respective organs are not prepared. The Perinatal Life Support (PLS) consortium envisions a medical device that can support the safe development of EP infants outside the womb by preserving the innate fetal cardiorespiratory physiology *ex vivo*. Part of this artificial womb system is a liquid-based environment that provides the EP infant with oxygen and nutrients using an artificial placenta. LifeTec Group is developing an *ex vivo* organ model to evaluate prototype suitability in ensuring prolonged viability of individual organs. To develop this model, a porcine liver and blood are



procured at a slaughterhouse and reperfused at LifeTec Group laboratory. Antibiotics are used to ensure blood sterility. During reperfusion, several vital parameters are monitored: O<sub>2</sub>, PO<sub>2</sub>, CO<sub>2</sub>, blood flow and blood pressure. Furthermore, the setup allows for measuring of hemoglobin, blood metabolites (e.g., lactate) and hemolysis and sterility are controlled periodically. In this study we develop a prototype organ perfusion system aiming to keep an organ in a functional state for prolonged time (up to 12 hours). The system will be used to research vascular cannulation techniques and organ (development) responses. Further research will focus on longer perfusion time and automatization of the processes needed to keep hemodynamic values and blood parameters in an acceptable range. Overall, the integrated PLS system will allow major progress towards translation for an urgent medical need, where new solutions are lacking as preclinical models are inadequate and clinical trials not feasible.

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**Presentation:** Poster

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## Modulation of P2X7 receptor using quercetin: Role in COVID-19 inflammation management?

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**Background and Objectives:** The world has been facing the SARS-CoV-2 pandemic for more than one year. Till now, there is no curative treatment for COVID-19 disease. Clinical observations highlighted a cytokine storm leading to multiorgan damage in severe COVID-19 patients. At the cell level, cytokine storm is triggered by inflammasome activation, itself triggered by the activation of the P2X7 purinoreceptor. Molecular docking studies revealed that the flavonoid quercetin could be a good candidate for the management of SARS-CoV-2 due to its antioxidant and anti-inflammatory properties, with no side effects when ingested. Our objective was to study the modulation of P2X7 receptor and cytokine release by quercetin on a pulmonary *in vitro* model and to check the effect of quercetin on SARS-CoV-2 infection.

**Materials and Methods:** We performed our study with the human bronchial BEAS-2B cell line. The cells were preincubated with quercetin (6.25-50 µM) for 20 minutes, then the cytokine storm was induced using poly(I:C), a widely used viral mimetic. P2X7 receptor expression was measured using Western Blot technique and its activation using the YO-PRO-1 assay. The release of IL-6 and IL-8 proinflammatory cytokines were respec-

tively quantified using Lumit<sup>TM</sup> and ELISA techniques. Lentivirus Construct Core expressing SARS-CoV-2 Spike protein from Brown University was used to mimic SARS-CoV-2 infection.

**Results:** Quercetin at 50 µM reduced the release of IL-6 and IL-8 cytokines induced by poly(I:C) and counteracts the P2X7 receptor activation at 50 µM. Quercetin at 50 µM had no effect on pseudovirus SARS-CoV-2 infection.

**Discussion:** Quercetin reduced P2X7 receptor activation and acted as an anti-inflammatory compound against the viral mimetic poly(I:C). Quercetin didn't demonstrate any significant effect against pseudovirus SARS-Cov-2. This flavonoid didn't seem to act specifically on SARS-CoV-2 infection process, nevertheless quercetin appeared to be an interesting modulator of P2X7 receptor and inflammation.

**Presentation:** Poster

1058

## A 3D alveolar *in vitro* model for the prediction of chemical respiratory sensitizers and irritants

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Respiratory allergic diseases have developed to a global health problem, resulting in high morbidity and mortality. The immunological mechanisms leading to skin sensitization are well characterized, while the mechanisms involved in respiratory sensitization caused by chemical compounds are still not fully unraveled. The available validated methods for the identification of skin sensitizers fail to distinguish respiratory from skin sensitizers.

We aimed to test the capacity of our 3D-alveolar *in vitro* model for the prediction of respiratory sensitization (Chary et al., 2019). The model is built on a permeable membrane using cell lines: alveolar epithelial cells (A549) are seeded on the apical surface and endothelial cells (EA.hy926) on the basolateral side. Macrophage-like cells (PMA-differentiated THP-1 cells) and dendritic-like cells (THP-1) are distributed on the luminal surface of the epithelial, respectively beneath the endothelial cell monolayers. The 3D setup allows the exposure to chemicals under realistic conditions, at the air-liquid-interface, and the development of a tissue-like microenvironment by cell-to-cell direct communication and indirectly through secreted messenger molecules.

A set of 12 chemicals was tested to verify the ability of the model to discriminate between respiratory and skin sensitizers, and irritants. Viability was measured 24 hours post-exposure to obtain a dose-response curve for the calculation of the dose inducing a 25% reduction of cell viability (CV75) for each compound. After exposing the model at the CV75 dose of the select-



ed compounds, the expression of specific cell surface markers was measured in the dendritic-like cells.

Our data show that the model can discriminate respiratory sensitizers from irritants. The model correctly identifies piperazine as a respiratory sensitizer, chemical which has been reported as false negative in other assays. In a next step additional sensitizers, irritants and innocent chemicals will be tested to further evaluate false negative and false positive results.

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**Presentation:** Poster

1059

## The chicken chorioallantoic membrane as a pre-screening method for monitoring biocompatibility of porous biopolymer scaffolds

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The chorioallantoic membrane (CAM) is an extraembryonic membrane that is commonly used to study angiogenesis, and its inhibition in response to tissues, cells, or soluble factors (Mangir et al., 2019). We studied the angiogenic response of the chicken CAM (*Gallus gallus domesticus*) after the implantation biopolymer composite prepared on the base of polyhydroxybutyrate and chitosan. This study described an approach to detect the formation of blood vessels inside biopolymer scaffolds using the CAM as an *in vivo* alternative animal model. Differences in the angiogenic response of CAM was observed depending on the addition of vascular growth factors (VEGF-A, FGF-2), and FGF-2 inhibitor (SU5402).

On embryonic day 7 (ED7), the tested biomaterial was placed on the CAM alone/or soaked with growth factors VEGF-A and FGF-2 at an application dose of 25 ng. Similarly, scaffold was soaked with FGF-2 inhibitor SU5402 in concentration 5 mM and placed on the CAM. Three days after the implantation, the vascular reaction was documented using stereomicroscopy. We observed and evaluated formation of vessels in surrounding area of the scaffold as well as inside of the implant by WGA marker of endothelial cells and KUL-01 marker of macrophages.

The morphological and histochemical analysis showed the highest angiogenic potential in untreated scaffold (77.51%) compared to soaked scaffolds with pro-angiogenic factors (VEGF-A – 75.11%, FGF-2 – 69.82%). The weakest angiogenic potential was observed in scaffolds soaked with FGF-2 inhibitor SU5402 (19.69%). Gene expression of pro- and anti-angiogenic markers followed similar results. VEGF-A promotes angiogenesis extensively in untreated scaffolds, while FGF-2 caused less effective proangiogenic response. FGF-2 anti-angiogenic inhibitor, SU5402 partly weakened the angiogenesis in both untreated and treated scaffolds (VEGF-A, FGF-2).

The CAM is a rapid way to specify biocompatibility and angiogenic potential of porous biomaterials intended for use in the field of tissue engineering with respect to the 3Rs.

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**Presentation:** Poster

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## Effect of cooling as an anesthetic method for zebrafish (*Danio rerio*)

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Zebrafish is a popular model organism in many research areas due to its high fecundity, the convenience of handling, availability of genetic information and transparency of the embryo (Lieschke et al., 2007). The commonest method of fish anesthesia is the use of MS 222, which is expensive and can cause toxicity at higher doses. Some anesthetics may interfere with the sample processing (ex. Clove oil) and some may cause adverse effects on tissue (ex. Lidocaine) (Collimore et al., 2014). Rapid cooling is an alternative for such anesthetics and this study aims to determine the effect of cooling on adult zebrafish. Both gradual cooling effect starting from room temperature (25°C) and acute cold exposure (10°C) were examined using 24 adult zebrafish. Time and temperature at the anesthetic phase and recovery phase were obtained. In gradual cooling, loss of balance initiated after 2 minutes at 16.5 (± 0.5)°C, loss of fin movements after five minutes at 10 (± 0.5)°C and the loss of opercular movement was observed after 6.5 minutes at 9 (± 0.5)°C. Comparatively, five zebrafish used in acute cold exposure at 10°C were anaesthetized within one minute. In both methods after transferring to fresh water at 25°C, rapidly recovery (initiation of opercular movement, fin



and body movements) occurred in two minutes. After the gradual cooling method, no mortality was observed while one death was reported from the acute cold exposure method. In conclusion, cooling can be practiced as a cost-effective, minimum risk and rapid anesthetic method for short term anesthesia.

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**Presentation:** Poster

1062

## Developing a new approach to assess crop protection chemical safety that minimizes reliance on vertebrate testing and protects human health and the environment

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Efforts to reduce the use of vertebrate studies and improve regulatory science for the assessment of agrochemicals challenge crop protection companies to develop new approaches for registration of new active ingredients (AIs) that ensure no unreasonable risk to human health and the environment. To determine the scope and direction of a project, Syngenta developed a problem statement in collaboration with regulatory agency scientists: "Develop, demonstrate and implement a modern scientifically-sound strategy that applies appropriate and flexible exposure and effects characterization without chemical specific vertebrate tests to robustly and reliably address risk, uncertainties, and deficiencies in data, and its interpretation, with the same or greater confidence to that provided by the currently accepted test guidelines and meets the regulatory needs of the agencies". To address this problem, a project was initiated to create a case study applying existing conceptual frameworks based on modern scientific approaches 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, determine whether these frameworks meet risk assessment needs for human health and vertebrate ecotoxicology, and to identify where further development of new approaches for compound-specific data generation would be required. To maximize the reuse of existing data, we select-

ed a new herbicidal AI with an established mode of action having many exemplars for which the toxicology and ecotoxicology is well characterized. The presentation will demonstrate that these data can be curated and analyzed to provide an appropriate human health and vertebrate ecological hazard characterization for the purpose of risk assessment of a new AI, without the generation of new vertebrate data.

**Presentation:** Poster

1064

## Toward regulatory acceptance of recombinant Factor C based assays in the United States

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All vaccines and injectable therapies must be tested for fever-inducing contaminants, called pyrogens, before administration to humans. Bacterial endotoxins testing (BET) provides rapid and quantitative analysis of the most common pyrogen risks. The frequently used *Limulus Amoebocyte Lysate* (LAL) assay relies on the clotting cascade of zymogen proteases isolated from horseshoe crab blood. Efforts to modernize the production of BET reagents have yielded a synthetic source of the key enzyme in the pathway, Factor C, via recombinant production. Notably, the BET using recombinant Factor C (rFC) eliminates the non-specific pathway that causes artificially increased endotoxin measurements common to LAL assays, avoids variation within LAL, helps avoid supply chain issues associated with relying on a single species, and helps companies meet ethical commitments to reduce animal testing.

Ongoing efforts over the past two decades to evaluate rFC-based assays have demonstrated that recombinant assays give equivalent results to traditional LAL assays in response to prepared LPS as well as naturally occurring endotoxins. This has led to the Pharmacopeial adoption of rFC as a compendial BET method in Europe as well as FDA-approval of parenteral drugs based solely on rFC data in the United States. In an effort to work toward global harmonization, global pharmacopoeias and health authorities have an opportunity to remove barriers to rFC use by modifying published guidelines and guidance to allow sponsors to submit rFC data for BET requirements without additional evaluation hurdles. In the United States, opportunities exist for the United States Pharmacopeia and the Food and Drug Administration.

**Presentation:** Poster



1074

## Policy initiatives for integrating new approach methodologies for testing pharmaceuticals in the United States

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New approach methodologies (NAM) that are expected to better protect public health than traditional nonhuman animal-based approaches are at the forefront of toxicology testing. Many NAMs have been commercialized, and both private and publicly funded scientists are working to develop a multitude of additional NAMs that utilize human cells and tissues. In order to support NAM integration into regulatory decision-making for drug development, policies outlining a drug sponsors requirements and expectations must clearly allow for NAM use. This level of regulatory certainty is critical because traditional animal studies are otherwise ingrained in regulatory policy and industry practice.

Multiple activities in the United States are moving toward integration of new approaches. FDA's Innovative Science and Technology Approaches for New Drugs (ISTAND) is a pilot program that provides an evaluation pathway for NAMs that may become acceptable for regulatory use under the program. A method qualified for a context of use under IStand can be included in regulatory submissions for the context of use without the need for FDA to reconsider and reconfirm its suitability. Opportunities remain for updating the regulatory framework. For example, many regulations require nonclinical animal data. To account for evolving NAM development, requirements should be neutralized by changing references from "in vivo" and "animal" to "nonclinical," which encompasses *in vivo*, *in vitro*, and *in silico* approaches.

**Presentation:** Poster

1075

## Challenges in developing an online animal ethics course

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Recent lockdowns as a result of the ongoing global COVID-19 pandemic forced many universities to convert face-to-face teaching to online platforms to deliver lectures and tutorials. The UNSW animal care and ethic course was offered as a blend of face-to-face lectures, workshops and an introductory hands-on session on common procedures in laboratory mice and rats. This course was designed to introduce the arrangements that support the eth-

ical and legislative oversight of the use of animals for scientific purposes in Australia and to raise awareness of the scientific and ethical issues that need to be taken into account and the practical considerations to ensure high standards of animal welfare and scientific integrity.

The Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013) details the responsibilities of all people involved in the care and use of animals and mandates appropriate education, training, and assessment of competence of investigators be they researchers, teachers, or students (Rose and Grant, 2013). As animal-based research was still ongoing during the lockdown in Sydney, there still existed demand for the animal care and ethics course which could not be delivered in its current format due to pandemic-related restrictions. As such the existing course was converted into online modules to comply with the education and training requirements of the Australian code.

This presentation will discuss the process of converting a face-to-face course to an online platform within a restricted time frame. It will identify the problems and challenges encountered during development and after the release of the online modules. We will also discuss how the face-to-face hands-on training session of the course was conducted during physical distancing restrictions, and how it inadvertently led to a refinement in the practical training component of the animal care and ethics course.

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**Presentation:** Poster

1076

## Systems vaccinology enables to evaluate vaccine safety and quality *in vitro*

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Vaccines are the most effective tool to prevent and control infectious diseases in humans and remain a major combatting tool for newly emerged infectious diseases such as SARS-COV-2. Recent advances in vaccinology, combined with molecular biology and immunology, enable us to develop well-designed vaccines to induce target-oriented immunity without using conven-

tional vaccine production platforms, such as the inactivation of live pathogens and splitting their components. On the other hand, assessment of vaccine safety at the preclinical studies and lot to lot quality evaluation after the licensure using animal model have not changed since these testing methods were introduced 50 years ago. Our laboratory has been committed a vaccine safety and assuring the lot-to-lot consistency of vaccines as a national control laboratory. Herein, we introduced our new assessment method for vaccine safety and quality control (Mizukami et al., 2014). Genomic profiling approaches enable us to identify potent biomarkers (BMs) for evaluating the safety and efficacy of influenza vaccines and adjuvants administered via any *in vivo* injection route in mouse models (Sasaki et al., 2020, 2018a,b,c). Some parts of BMs could be applied or extrapolate for humans using *in vitro* human peripheral blood mononuclear cell models (Sasaki et al., 2018a,b,c) and *in vivo* humanized mouse models. Recently, we have also identified cell line that could recapitulate BM expression after the vaccination in animal model and evaluate vaccine quality using BM expression. Taken together, our findings could allow us to bring next-generation *in vitro* safety and quality evaluations of vaccines and adjuvants.

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**Presentation:** Poster

1078

## Dynamic *in vitro* assessment of lung epithelial cells under mechanical strain

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A bioreactor capable of simulating breathing motions was developed to enhance the physiological relevance of lung cell models and to investigate the effects of aerosolized nanoparticles on air-liquid interface (ALI) of the lung cells. DALI (Dynamic model for Alveolar Interface) is a cutting-edge device developed within the framework of PATROLS (EU H2020) to study lung cell behavior under breathing conditions (Nossa et al., 2020). It features an ALI, cell culture media flow to mimic blood flow in the lower compartment, a permeable membrane stretching, and an aerosol deposition system for nanoparticle exposures. The membrane was fabricated by electrospinning made of Bionate<sup>®</sup>. This highly biocompatible and stretchable material replicates the alveolar basement and ensures membrane flexibility, which is needed to mimic the cyclic motion during breathing (Agarwal et al., 2008). To optimize cell growth, *in vitro* experiments were performed using the human alveolar epithelial cell line A549 on both uncoated and rat-tail collagen (RTC) coated membranes under submerged conditions. With the coated membranes the A549 cells achieved a higher level of confluency. Optimal cell growth was discovered when cells were cultured for 1 day submerged and 2 days at ALI on RTC coated membranes. In conclusion, A549 cells are well adapted to culture in a DALI-bioreactor, and a confluent cell layer was obtained that remained stable under stretching conditions that mimicked breathing. A549 cells will be exposed to aerosolized DQ12, TiO<sub>2</sub> (NM-105), LPS (positive inflammatory control), and water (negative control) in a DALI-bioreactor with and without stretching/breathing in future experiments.

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**Presentation:** Poster



1079

## An inverted *in vitro* triple culture model of the healthy and inflamed intestine: Adverse effects of polyethylene particles

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Advanced *in vitro* models possess great potential as an alternative to animal studies and can be adapted and developed to address highly specific research questions. Previously, we established a triple culture transwell model of the healthy and inflamed human intestine composed of enterocytes (Caco-2), mucus-producing goblet cells (HT29-MTX-E12) and macrophages (THP-1) for the investigation of ingested particulate toxicants. Polyethylene (PE), one of the most produced polymers and most abundant type of microplastic particles in the environment, food and water, has a density of  $\sim 0.95$  g/cm<sup>3</sup> and is therefore buoyant in cell culture medium. However, physical interaction between cells and particles is pivotal when investigating particle toxicity *in vitro*. Thus, we established a spatially inverted modification of the triple culture transwell model, which allows direct contact between buoyant particles and cells to study the intestinal effects of polymeric particles with a density of  $< 1$  g/cm<sup>3</sup> *in vitro*. We validated this model against the original model in regular orientation using the enterotoxic, non-steroidal anti-inflammatory drug diclofenac and subsequently assessed the cytotoxic and pro-inflammatory effects of PE microparticles. The results show that the inverted model exhibits the same distinct features as the original model in terms of barrier development and inflammatory parameters. Treatment with 2 mM diclofenac causes severe cytotoxicity, DNA damage and complete barrier disruption in both models. PE particles induced cytotoxicity and pro-inflammatory effects in the inverted model, which would have remained undetected in conventional *in vitro* approaches, as no effect was observed in non-inverted control cultures.

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**Presentation:** Poster

1080

## Comparison of the effects of engineered nanomaterials in simple versus complex *in vitro* models of human intestine

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With growing knowledge on the importance of the intestine for overall health, increasing effort is invested in the development of advanced intestinal *in vitro* models to study the effects of engineered nanomaterials (ENM) after ingestion. Whereas highly sophisticated models are available, the necessity of their individual building blocks and selected endpoints are rarely determined.

We studied silver (Ag-PVP) and titanium dioxide (TiO<sub>2</sub>) ENM in four *in vitro* models of increasing complexity: proliferating monocultures of Caco-2 and HT29-MTX-E12 (E12) cells, Caco-2/E12 co-cultures, and triple cultures with THP-1 cells in healthy and inflamed state (Kämpfer et al., 2021). Acute exposures over 24h were compared to repeated exposures over 5 days. Adverse effects were only observed in proliferating monocultures and were more substantial for Ag-PVP. Even high concentrations of ENM did not impact cell viability, cytokine release or DNA damage in advanced models. Neither the inflammation-like state nor the repeated exposure resulted in enhanced adverse effects of the tested ENM.

The results underline how the choice of parameters can substantially affect the outcomes, at least for the limited number of endpoints and ENM tested. The inclusion of additional parameters and use of other ENM might affect this (Llewellyn et al., 2021). While proliferating monocultures could facilitate a time and cost-effective approach for the screening or prioritizing of ENM, advanced models are likely more suitable for mechanistic or AOP-related research.

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**Presentation:** Poster

1081

## Gap analysis of effect-directed monitoring tools for risk assessment of drinking water

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Providing clean drinking water remains a challenge. Although safe drinking water is characterized as potable for human consumption and contains non-toxic levels of concentrations of chemicals, potential adverse effects of chemical mixtures and unknown chemicals are impossible to assess with the current regulated analytical methods. Effect-directed monitoring tools or bioassays conform to the 3Rs principle and are cost-effective methods that help overcome the challenge of providing safe drinking water. In this study, we gathered the available *in vitro* bioassays that are used in studies assessing water quality. Moreover, a gap analysis was performed to highlight the missing toxicity endpoints to monitor the potential adverse effects associated with prioritized chemicals in drinking water. The analysis shows that some toxicity endpoints are not well covered by bioassays, such as neurotoxicity, nephrotoxicity, cardiotoxicity, teratogenicity, developmental toxicity, hematotoxicity, reproductive toxicity. In a further step, we gathered the available bioassays for risk assessment that could be implemented to extend the monitoring of human toxicity and human embryonic toxicity. The result show that various *in vitro* tests developed for chemical risk assessment, such as the embryonic stem cell test and multi-electrode arrays assay, may complete the battery of bioassays in the context of water quality monitoring.

**Presentation:** Poster

1082

## A novel alternative test platform for real-time monitoring of TEER values in human epithelial tissue models

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Robust and reliable human epithelial tissue models can accelerate the use for early screening testing in pharmaceutical, cosmetic, and chemical industries as an alternative *in vitro* test method (Bas et al., 2021). Epithelial cells are typically assembled on semi-permeable inserts consisting of two-chamber compartments. Transport studies and drug efficacy tests are assessed with transepithelial resistance (TEER) measurements of tight junction formation along with permeability assays. However, both assays require a handling of the cells outside the cell incubator and is time consuming and operator dependent. Aim of this project is to use a smart insert (SiMPLI SSI<sup>®</sup>) (Jud et al., 2015) featured with integrated non-invasive electrodes to monitor on-line the impedance, as biomarker of tissue permeability, and an ultrathin permeable support to enhance the physiology of the cell growth environment. We compared the cell growth of different human epithelial cell lines, i.e., two lung cell lines (A549, Calu-3), a retinal cell line (ARPE-19), and an intestine cell line (Caco-2) on the passive version of the SiMPLI insert and compared cell growth and TEER values with cells grown on conventional polyethylene terephthalate (PET) inserts. The data revealed a comparable cell growth morphology and increase in impedance on both insert types. Calculation of absolute TEER values is ongoing. In addition, apparent permeability (Papp) values will be used. We will transfer the optimized tissue design onto the smart version of the SiMPLI inserts to demonstrate the superiority of the SiMPLI-CoMPLI SSI<sup>®</sup> system as a reliable platform for real-time monitoring of TEER values in epithelial tissue models relevant for early drug screening or testing of chemicals. We are paving the way to making quantitatively reliable the use of impedance as parameter to assess human epithelial tissue permeability and, with the same set-up, to enable the insight into the polarity, the adhesion and the proliferation of cells constituting it.

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## Performance of the GHS mixtures equation for predicting acute oral toxicity

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The United Nations Globally Harmonized System for Classification and Labeling (GHS) provides a mathematical approach to estimate the acute oral toxicity of a mixture based on the combined toxicities of its individual components. We evaluated how well toxicity values calculated using the GHS formula, and the corresponding U.S. Environmental Protection Agency (EPA) and GHS hazard categories, agreed with those obtained from *in vivo* acute toxicity studies of the same formulations. Data were compiled for approximately 700 formulations submitted for pesticide registration including agrochemical and antimicrobial product formulations, most of which were classified in the less hazardous EPA Categories III and IV and GHS Categories 4, 5, and Not Classified (NC). The extent to which more toxic formulations might be underpredicted by the mixtures equation could not be addressed with full confidence. Overall concordance was between 55% to 82% depending on the classification system and the ranges of LD50 values used. Most discordant results were associated with substances with a limit test LD50 > 2000 mg/kg or a measured LD50 between 2000 and 5000 mg/kg, which were predicted using the mixtures equation as having minimal toxicity LD50 > 5000 mg/kg. Therefore, most of the discordance observed may be of lesser concern than if more toxic substances were underpredicted. Our results suggest the mixtures equation is a promising approach for identifying substances that would not be expected to induce toxicity. The lack of more toxic formulations in the data set preclude us from reaching definitive conclusions across the spectrum of hazard categories.

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*Disclaimer:* The views expressed above do not necessarily represent the official positions of any federal agency.

**Presentation:** Poster

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## Chronic cardiac contractility assessment of human iPSC-derived cardiomyocytes in a pro-maturation environment for preclinical cardiac safety and toxicity studies

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Undetected cardiovascular side effects of newly developed drugs entering clinical phases are one of the major reasons for costly late-stage drug attrition. This issue is partly generated through preclinical cardiac safety and toxicological approaches using animal models that are not 100% translatable to humans. Due to the advantages of direct species applicability as well as cost and time-effectiveness, human cell-based assays provide an ideal alternative.

Human iPSC-derived cardiomyocytes (hiPSC-CM) are predominantly used in modern cardiac safety and toxicity assays due to their reproducibility and low ethical concern. Nevertheless, their premature phenotype causes issues concerning non-physiological responses to positive inotropic compounds. Another limiting factor is the primary application of hiPSC-CMs for acute testing with timescales ranging from minutes to hours, partly due to the inability of common cell-based assays to analyze cellular behavior reliably over prolonged periods of time.

Here we describe the high-throughput 96 well FLEXcyte technology and its positive effect on hiPSC-CM maturation as well as its applicability to assess cardiovascular safety and toxicological questions on acute as well as chronic level. hiPSC-CMs treated with positive inotropic compounds isoproterenol, S-Bay K8644 and omecamtiv mercabil displayed the desired adult-like CM responses when plated on FLEXcyte 96 plates, proving the pro-maturation effect of a physiological environment. A comprehensive inotropic toxicological study was also conducted with 18 anthracyclines and tyrosine kinase inhibitors (all with well-known toxicological profile) over a period of 5 days to evaluate chronic cardiotoxic side effects on hiPSC-CMs.

Our results indicate that the FLEXcyte technology enables the assessment of physiologically relevant inotropic effects of hiPSC-CMs on an acute and chronic level for safety and toxicological studies that will diminish discrepancies between preclinical and clinical studies due to direct species applicability.

**Presentation:** Poster



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## Contactless body temperature assessment for determining humane endpoints in the cecal ligation and puncture model of sepsis

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The severity of experimental sepsis raises welfare concerns in animal research. Humane endpoints are therefore paramount, but lack of objective criteria for euthanasia can lead to experiments being terminated either too soon (wasting animals' lives) or too late (causing unnecessary suffering). Severe hypothermia has been proposed for signaling humane endpoints in sepsis research. Temperature variations are, however, model-dependent, thus case-by-case determination of cut-off points is warranted. The goal of this study was to identify early temperature cut-off points that by themselves or combined with other clinical signs (e.g., weight variation) could robustly signal non-recovery in murine models of sepsis.

Abiding to the principle of Reduction, we collected data from animals already destined to be used by ongoing research work in other projects. We followed a cohort of C57BL/6 mice ( $N = 27$ ) subjected to sham surgery or experimental sepsis by severe cecal ligation and puncture (CLP) and a cohort of mice ( $N = 24$ ) with mid-grade severity CLP-induced sepsis. The first were monitored 4 times per day, while the latter were monitored thrice daily for 10 days post-surgery or until reaching a predefined humane endpoint based on a clinical score. This included assessment of subcutaneous temperature (read from thermosensitive PIT tags) and surface temperature (thermal imaging).

Receiver operating characteristic (ROC) curve analysis shows predetermined cut-off points for temperature drop and percentage of weight loss robustly predict non-recovery stages (with high sensitivity and specificity) in the severe model of CLP. However, body surface temperature assessment by thermal imaging did not allow assessing non-recovery stages, likely from cages being kept on a heating pad, following recommendations of the animal facility. These results suggest subcutaneous temperature and weight loss are relevant markers to assess disease progression and non-recovery stages in a severe CLP sepsis model, and a combination of the two parameters may further refine welfare assessment.

**Presentation:** Poster

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## Standardizing developmental toxicology study extractions using automated application of ontologies

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Extraction of toxicological data from primary sources is a central component of systematic reviews in human health risk assessment. Data obtained from disparate sources must be standardized to ensure that endpoints are identified in a harmonized manner. This standardization process, which can require large labor resources if done manually, is critical to downstream data analyses such as calculating chemical-specific effects, establishing reference datasets for validation of new approaches, and computational modeling purposes.

To expedite the process, and reduce overall level of effort required, we developed a Python script that automates application of pre-existing ontologies and controlled vocabularies to extracted endpoints. Our approach created a harmonized controlled vocabulary crosswalk comprising Unified Medical Language System codes, German Federal Institute for Risk Assessment DevToxDB ontology terms, and Organisation for Economic Co-operation and Development endpoint vocabularies. The crosswalk was applied to roughly 36,000 extractions from prenatal developmental toxicology studies conducted by the National Toxicology Program and 6,400 extractions from prenatal developmental toxicology studies submitted by registrants to the European Chemicals Agency (ECHA).

Our script automatically applied standardized terms to 76% of the NTP extracted endpoints and 60% of the ECHA extracted endpoints. About half (53%) of the standardized terms required manual review after automation to ensure accuracy. Extracted endpoints that were not mapped to standardized terms were too generalized (e.g., "number of fetuses with abnormal organ") or required human logic to find an adequate match. We estimate that our automated language standardization saved ~375 hours of time while still yielding a valuable computationally accessible dataset. This open-source approach can be applied to other developmental toxicology datasets or customized for other study types to leverage legacy datasets for use in modeling or other analyses.

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**Presentation:** Poster



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## High-throughput screening to predict hERG inhibition

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The human ether-a-go-go related gene (hERG) potassium channel, a member of voltage-gated potassium channels, plays a pivotal role in cardiac rhythm regulation, especially in the repolarization of cardiac action potential. Inhibition of hERG channels can lead to a prolongation of the QT interval which, in the severe case, triggers torsade de pointes arrhythmia and can progress to ventricular fibrillation and sudden death. Environmental toxicants have the potential to contribute to the pathophysiology of complex diseases, but the underlying mechanisms remain obscure. To date, more than 100,000 chemicals have been introduced into commerce with limited toxicological testing. An evaluation of the effect of environmental chemicals on hERG channel function can help inform the potential public health risks of these compounds. To assess the effect of environmental chemicals on hERG channels, the US federal Tox21 program has screened a collection of 9667 chemicals using a cell-based thallium-influx assay in U2OS cells stably expressing hERG in a quantitative high-throughput screening (qHTS) format. The chemical results in the hERG qHTS assay were characterized using a set of 1D/2D molecular descriptors and physicochemical properties, Self-Organizing Maps (SOM) and hierarchical clustering. Statistical machine learning approaches were applied to build quantitative structure-activity relationships (QSAR) models to predict the probability of a chemical to inhibit hERG in this thallium flux assay, applying both classification and regression techniques. Models were compared with existing QSAR hERG models and dataset. The evaluation of performance criteria of generated models revealed that Random Forest model outperforms other models and demonstrated 0.907 cross-validated, 0.928 test set accuracy and equivalent performance against external test sets. This tiered clustering and predictive modeling approach facilitates detection of environmental chemicals that merit more extensive evaluation for cardiotoxicity and provides useful structural information that could be applied to predicting the potential for new chemical entities to inhibit hERG.

**Presentation:** Poster

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## Systematic evidence mapping of research on environmental exposures and cardiovascular disease

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Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels that represent a leading cause of mortality in the United States and worldwide. Various factors influencing CVD have been relatively well-characterized, such as lifestyle choices, genetic factors and off-target pharmaceutical effects. Another potentially significant but underappreciated risk factor contributing to the development and severity of CVD is environmental exposure to chemicals that may interfere with critical CV targets and pathways. The heart and vascular system have been shown to be vulnerable to multiple environmental agents including pesticides, flame retardants, polycyclic aromatic hydrocarbons (PAHs), plasticizers, air pollutants, arsenic, cadmium, lead, and there is mounting evidence that long-term environmental chemical exposure plays a significant role in progression of CVD. To better understand the landscape of environmental chemical influence on CVD, we developed a scoping review to systematically identify and categorize research reporting potential associations between environmental exposures and adverse cardiac outcomes. A comprehensive search was conducted in Pubmed, Scopus, and Web of Science that retrieved over 200,000 references. Given the particularly large number of references, iterative artificial intelligence algorithms were leveraged to prioritize and support manual title and abstract screening in Distiller, and machine learning approaches were used to facilitate categorization of references that reported data on cardiovascular outcomes after exposure to an environmental agent. Relevant references were characterized by evidence stream (human, animal, or *in vitro* exposure), study design, exposure, and major CV outcomes. An interactive evidence map was prepared to enable researchers to explore data rich and data poor areas in the literature by cardiovascular outcomes, environmental exposures and other key factors. This map will inform evidence-based decisions on the identification, selection, and prioritization of assay platforms and environmental chemicals that will be used by the DNTP to evaluate cardiovascular toxicity.

**Presentation:** Poster



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## Assessment of phototoxic potential of chemicals using the *in vitro* 3D-PT SkinEthic RHE test method

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Phototoxicity is defined as a toxic response elicited by topical or systemically administered photoreactive chemicals after the exposure of the body to environmental light. Photosafety evaluation of any new ingredients (pharmaceuticals or cosmetics) should be therefore conducted when any probabilities of human exposure might have occurred. In the context of animal testing ban, international standards for photosafety assessment have been adopted as guidance with the consideration to the use *in vitro* alternative methods.

The proposed 3D-PT SkinEthic™ RHE test method is based upon a comparison of the cytotoxicity of a chemical when tested with (+UVA) and without (-UVA) exposure to a non-toxic dose of UV light. Cytotoxicity is determined as reduction of mitochondrial conversion of MTT to formazan and a chemical is predicted to have a phototoxic potential if a decrease in viability exceed 30% when compared with the identical concentrations of the (-UVA) part.

The method has been progressed through a protocol optimization phase (with training and test chemicals sets). More than 30 chemicals (well-balanced set) were tested in at least a run. Analysis of the data identified the majority of non-phototoxicants as negative *in vitro*. On contrary, a significant decrease of cell viability was observed in treated tissues exposed to UVA, as compared with those non-exposed for the phototoxicants. Sensitivity and specificity were therefore high indicating that the method was able to discriminate efficiently between phototoxic and non-phototoxic products.

In conclusion, this study provides evidence that the 3D-PT SkinEthic™ RHE test method is capable of correctly predict the phototoxicity potential of chemicals. Therefore, the method supports international directives towards reducing and replacing the use of animals in chemical photosafety evaluations as well as the ongoing new OECD "In Vitro Phototoxicity: Reconstructed Human Epidermis Phototoxicity Test (RHE PT) Method" test guidelines.

**Presentation:** Poster

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## Lack of enforcement actions by USDA against research facilities using animals

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The U.S. Department of Agriculture (USDA) is responsible for inspecting animal research facilities to ensure compliance with the Animal Welfare Act (AWA) and corresponding regulations. To understand whether these inspections uphold animal welfare standards, we tracked the inspection reports posted on USDA's public search tool. Over 5000 inspections were completed at registered facilities in a 5-year period. Over 1000 non-compliant items (NCIs) were cited on the inspection reports, with approximately 14% of the NCIs being critical problems and approximately 2% being direct violations of the AWA. The NCIs involved multiple species, including dogs, cats, pigs, non-human primates, rabbits, and others. While some of the cited laboratories had one-time violations, others were repeat violators that had no enforcement action taken against them. For example, one facility had 20 cited violations and as of yet, this facility has faced no consequences for these violations. This is one of the more extreme examples, but there are many other facilities that have repeat AWA violations.

Enforcement action is rarely taken against research facilities, despite the large number of violations cited. Here, we look at how research facilities that violate the AWA are permitted to continue research and testing on animals at their facilities with little or no enforcement action taken by USDA. We also examine the lack of clarity around what qualifies a laboratory to have enforcement action taken against it by the USDA. Additionally, we offer recommendations to improve enforcement for noncompliant facilities and address the serious concerns for animal welfare that result from enforcement failures.

### Reference

USDA's public search tool of inspection reports. <https://aphis-efile.force.com/PublicSearchTool/s/inspection-reports>

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## ALT4EI: Determination of eye irritating potential of 59 chemicals using EpiOcular™ time-to-toxicity neat and dilution protocols

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Determination of acute eye irritation potential is part of international regulatory requirements for testing of chemicals. Objective of ALT4EI (ALternatives for Eye Irritation) project was to confirm the testing strategy developed in CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy) project. These projects focused on development of tiered testing strategies for eye irritation assessment for all drivers of classification and evaluation of whether the test methods can discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling for Category 1 (Cat 1) and Category 2 (Cat 2). In CON4EI project, a new testing strategy for EpiOcular time-to-toxicity was developed, the sensitivity for predicting GHS Cat 1 and GHS Cat 2 chemicals was 73% and 64%, respectively and the very high specificity of 97% was maintained. None of the Cat 1 chemicals was underpredicted as GHS No Category. Plus the goal of ALT4EI project was to fill remaining data gaps and strengthen the data set. A new set of 59 chemicals (41 liquids:(un)diluted, and 18 solids) was tested using the reconstructed human cornea-like epithelium, EpiOcular, in two EpiOcular time-to-toxicity tests (neat and dilution). The set of chemicals contained 32 chemicals not requiring classification (No Cat) and 27 chemicals requiring classification (16 Cat 2 and 11 Cat 1). The chemicals were tested blinded in two independent runs by MatTek IVLSL. In this study, a testing strategy to achieve optimal prediction for all three classes developed in CON4EI project (combining the most predictive time-points of both protocols and which tests liquids and solids separately) was used. Using the CON4EI testing strategy, we were able to identify correctly 63.6% of the Cat 1 chemicals, 56.6% of the Cat 2, and 76.6% of No Cat chemicals. Reproducibility between both runs was 88.7%. The combination of the EpiOcular time-to-toxicity neat and dilution protocols seems to be promising in integrated testing strategy (ITS) for eye irritation assessment.

**Presentation:** Poster

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## Cooperative training is an opportunity for refinement that eliminates restraint as a source of variation and confounding to improve the predictive value of primate models

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Primates have been essential in unlocking novel immune mechanisms that have driven the development of groundbreaking new therapies for infectious disease, transplantation, and autoimmune disorders. As a result, primates have served as critical “gatekeepers” in safety and efficacy evaluations of vaccines and immunotherapies prior to implementation in clinical studies. Experimental design that closely represents the intended clinical situation maximizes likelihood of successful translation. The use of primates in safety and toxicology studies often requires frequent and intensive handling to obtain required biological samples, perform thorough physical examination, and adequately manage the relevant disease state. Common sampling practice involves serial sampling from a chemically or capture (mechanically) restrained animal. While sedation-based sampling may protect researcher safety during studies of infectious disease and facilitate ease of sample collection, it typically has no clinical concordance, comes at a cost to animal welfare, and introduces potential confounders related to an elevated stress response that reduces accuracy in modeling the clinical situation and may complicate outcome interpretation. Consequently, important study specific endpoints related to stress, immune response, and safety may be influenced by model-imposed handling independent of disease or therapeutic effects, such that model design must be carefully considered to avoid misinterpretation of outcome measures. Additionally, sedative drug regimens used for chemical restraint introduce the risk of adverse events, such as vomiting and inappetence, which are outcomes that may be improperly attributed to the disease or therapy of interest and also affect welfare. There are significant effects of repeated sedation in primates on immune response, stress response, and adverse event prevalence in comparison with those trained to actively cooperate during equally frequent sampling events.

**Presentation:** Poster



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## International regulatory needs for acute toxicity data

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Chemical regulatory authorities worldwide consider acute systemic toxicity data to substantiate safety assessments; many regulatory agencies even require these data. Acute oral, dermal, and inhalation data are typically used to develop product hazard labels for consumer or worker protection and to assess risks from acute exposure to chemicals. Other uses for acute data may include setting occupational exposure levels, dose-setting for longer-term studies, and classifying mechanisms of action. Acute toxicity studies to generate regulatory information historically use animals to determine a lethal dose, which pose strong ethical and scientific concerns. To identify opportunities for regulatory uses of non-animal replacements for acute systemic toxicity tests, we reviewed acute systemic toxicity testing requirements for Brazil, China, Canada, Japan, the European Union, South Korea, and the United States. These jurisdictions also participate in the International Cooperation on Alternative Test Methods (ICATM). Our chemical sectors of interest for each jurisdiction were cosmetics and personal care products, consumer products, industrial chemicals, pharmaceuticals, medical devices, and pesticides. We found acute systemic toxicity data were often required for hazard identification rather than risk assessment. When animal methods were required, animal reduction methods and guidelines were typically recommended. However, for many jurisdictions and chemical sectors, non-animal approaches were not accepted. The most frequently acceptable non-animal approaches were test waivers when toxicity information was available from other studies. Understanding international regulatory requirements for acute systemic toxicity testing will inform ICATM's strategy for the development, acceptance, and implementation of non-animal alternatives to assess the health hazards and risks associated with acute toxicity.

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## Comparative toxicological analysis of an iPSC derived airway epithelium model with a primary bronchial airway epithelium model at an air liquid interface using TempoSeq

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The airway epithelium represents the main barrier between inhaled air and the tissues of the respiratory tract and is therefore an important point of contact with xenobiotic substances into the human body. Several studies have recently shown that *in vitro* models of the airway grown at an air-liquid interface (ALI) can be particularly useful to obtain mechanistic information about the toxicity of chemical compounds. However, such methods are not very amenable to high throughput since the primary cells cannot be expanded indefinitely in culture to obtain a sustainable number of cells. Induced pluripotent stem cells (iPSCs) have become a popular option in the recent years for modelling the airways of the lung, but despite progress in the field, such models have so far not been assessed for their ability to metabolise xenobiotic compounds and how they compare to the primary bronchial airway model (pBAE). Here we report a comparative analysis by TempoSeq<sup>®</sup> (templated oligo sequencing) of an iPSC derived bronchial airway model (iBAE) with a pBAE. The iBAE and pBAE were differentiated at ALI and then evaluated in a 5-compound screen with exposure to a sub-lethal concentration of each compound for 24 h. We found that despite lower expression of xenobiotic metabolism genes, that the iBAE similarly predicted the toxic pathways when compared to the pBAE model. Our results show that iPSC airway models at ALI show real promise for inhalation toxicity assessments with further investigation and development.

**Presentation:** Poster



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## Reconstructed human epidermis to prepare for animal test ban for cosmetics in Colombia

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The cosmetic industry in Colombia has been skyrocketing in the latest years, becoming one of the most booming in the region. The Law 2.047 of August 10<sup>th</sup>, 2020, issued by the National Congress prohibits the importation, manufacturing and sales of cosmetic products and their ingredients tested on animals, establishing a 4-year deadline for public and private sectors to adjust to this new reality, strengthening the need of robust alternative methods in Colombia. Skin irritation data is essential for safety evaluation of cosmetics. In Europe, validated alternative methods using reconstructed human epidermis (RHE) replaced the use of animal testing for cosmetics. Nevertheless, Colombia has not made relevant scientific advances to implement alternative methods. Here we describe a successful partnership between Belcorp, Colombian cosmetic company, and EPISKIN Brazil supplying SkinEthic™ RHE model.

For 10 years, Belcorp *in vitro* safety laboratory has been applying *in vitro* strategies to test the safety of cosmetic products and raw materials. These *in vitro* tests have been taking a strategic position through the implementation of international guidelines, beyond the design of own methodologies adjusted according to specific requirements.

Some biological and biotechnological materials are not accessible in Colombia, thus advances in the field of alternative methods in Brazil have been of huge influence to Colombian industry. The supply of SkinEthic™ RHE model by EPISKIN Brazil subsidiary is an important milestone to make such emblematic model available as alternative model for research, training and regulatory purposes.

Availability, training and dissemination efforts are crucial to prepare and anticipate the needs in terms of demand, creating an efficient network to widely deploy alternative methods throughout the country. With this recent legislative evolution, it is important to anticipate the implementation of alternative methods in order to be prepared when the law comes into force in 2024.

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## Research & Innovation

### L'OREAL

L'Oréal has devoted itself to beauty for over 100 years. With its unique international portfolio of diverse and complementary brands, the Group generated sales amounting to 26.9 billion euros in 2018 and employs 82 000 people worldwide.

Research & Innovation, and a dedicated research team of 3 993 people, are at the core of L'Oréal's strategy, working to meet beauty aspirations all over the world. L'Oréal's sustainability commitment for 2020 "Sharing Beauty With All" sets out ambitious sustainable development objectives across the Group's value chain.

Consumer health and safety is and has always been an absolute priority of the L'Oréal Group. Defending animals' welfare as well. To achieve these two objectives, L'Oréal conducts a very strict safety evaluation policy for its products. Starting by the development of the first models of reconstructed skins in 2019, L'Oréal has been a pioneer in the development and use of new alternative in vitro and in silico methods.

Thanks to this long term investment and conviction, L'Oréal stopped testing its products on animals in 1989, 14 years before required to do so by law. L'Oréal no longer tests its ingredients on animals neither tolerates any exceptions to this rule.

L'Oréal's commitment to ending animal testing is supported by the provision of reconstructed skin models thanks to 3 production Units through its subsidiary EPISKIN SA (based in France, China, Brazil), the development and validation of new alternative methods and the sharing of its scientific advances.

In 2017, the OECD adopted two new alternatives methods developed by the L'Oréal Research Laboratories, to evaluate skin allergy and eye irritation. Today, L'Oréal is committed to develop next generation of new safety assessment approaches alternative to animal testing to ensure product safety for consumers and environment and support innovation.

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#### PROCTER AND GAMBLE

Our brands are trusted everyday in millions of living rooms, kitchens, laundry rooms, and bathrooms – and have been family favorites from generation to generation for over 180 years. We know that to continue to be the brands people choose, we must continue to innovate high quality and safe ingredients. We also recognize that we must continue to evolve our approach to demonstrating safety.

We are committed to making animal testing obsolete. For more than 40 years, P&G has engaged in non-animal approaches and solutions. We have sponsored and contributed to all World Congresses on Animal Alternatives, including the first held in 1993 in Baltimore, Maryland. Over that time, P&G has invested more than \$420 million in developing non-animal alternatives, yielding more than 25 alternative test assays invented or co-invented by our experts. Many of these approaches have been accepted as the new standard in non-animal safety assessment used by academia, industry or regulatory authorities around the world. Some of them, like the Direct Peptide Reactivity Assay (DPRA), have been recognized with prestigious awards by animal welfare groups.

Yet there is more to be done. Therefore, we are a proud sponsor of #BeCrueltyFree, calling for an end to all animal testing of cosmetic products globally.

And we are pleased to sponsor the 11th World Congress to enable the sharing and reapplication of the latest progress in non-animal alternatives. Let's work together, because only together can we make our shared goal a reality: Making animal testing obsolete.

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#### UNILEVER

On any given day, 2.5 billion people use Unilever products. Our range of more than 400 brands give us a unique place in the lives of people all over the world. Seven out of every ten households around the world contain at least one Unilever product, and our range of world-leading, household-name brands includes Dove, Knorr, Lipton, Axe, Hellmann's and Omo. Unilever's purpose and business strategy are to make sustainable living commonplace.

We use a wide range of non-animal approaches to assess the safety of our products for consumers. We are committed to ending animal testing. Our leading-edge research has one clear purpose: to continue to develop new non-animal approaches that can guarantee that our products are safe, without any need for animal testing. As part of our commitment to ending animal testing, we have a growing number of brands that ensure that neither their products – nor the ingredients they use – are subject to animal testing by suppliers or by regulatory authorities. These brands' commitment to no animal testing is certified by animal welfare groups.

Our commitment to ending animal testing is under-pinned by our work since the 1980s in developing and using alternatives to animal tests for assessing safety, e.g. computer-based modelling and cell-based 'in vitro' methods. Unilever's framework for safety assessment is risk-based rather than hazard-based. This enables us to use a wide range of non-animal approaches to assess the safety of our products for consumers. We are making good progress in developing next generation (non-animal) risk assessment approaches for assessing new ingredients and share our scientific research on a dedicated

[Safety Science in the 21st Century website.](#)

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INTERNATIONAL**

#### HUMANE SOCIETY INTERNATIONAL

Humane Society International works to create a kinder, more humane world for all animals through science, education, advocacy and policy change. Our Research & Toxicology team includes scientists, regulatory and government affairs professionals who are active on the ground in the world's leading innovation economies. We work with industry and lawmakers to enact legislation that reduces reliance on animal testing in favor of best scientific practice and to implement bans on animal testing of cosmetics ([hsi.org/becrueltyfree](https://www.hsi.org/becrueltyfree)). We work with regulatory authorities and stakeholders to accelerate regulatory acceptance of animal-free safety assessment practices across multiple industry sectors ([animalfreesafety.org](https://www.animalfreesafety.org)). Our team also leads the BioMed21 Collaboration ([biomed21.org](https://www.biomed21.org)) to move medical research to embrace 21st century science.

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## Bronze sponsors



### ALTERNATIVES RESEARCH & DEVELOPMENT FOUNDATION

Established in 1993, ARDF promotes alternatives to the use of animals in biomedical research, testing and education. The foundation has awarded over \$3.5M through its Annual Open Research Grant program. It also sponsors scientific meetings and presents the Cave Award for outstanding achievements in advancing alternative methods. ARDF recently launched the Alternatives in Research Challenge, a program to focus science funding and prize money exclusively in the area of alternative methods for biomedical research.

### Beiersdorf

### BEIERSDORF

Beiersdorf is a leading provider of innovative, high-quality skin care products and has over 135 years of experience in this market segment. The Hamburg-based company has about 20,000 employees worldwide and is listed on the DAX, the German benchmark equities index. Beiersdorf generated sales of €7.2 billion in financial year 2018. Its product portfolio comprises strong, international leading skin and body care brands including NIVEA, Eucerin, Hansaplast/Elastoplast, and La Prairie.



### EPAA

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is an unprecedented voluntary collaboration between the European Commission, European trade associations, and companies from 7 industry sectors.

The partners are committed to pooling knowledge and resources to accelerate the development, validation and acceptance of alternative approaches to animal use in regulatory testing. The overall aim is the replacement, reduction and refinement (3Rs) of animal use in regulatory testing.



### IFRA

The International Fragrance Association (IFRA) is the representative body of the fragrance industry worldwide. Comprised of eight multinational companies, hundreds of small and medium-sized companies in 21 National Associations, and eight supporting members, IFRA's membership covers about 90% of the industry by production volume. We seek to promote the safe use of fragrance for everyone's enjoyment, working with regulators and promoting our flagship self-regulatory program, the IFRA Code of Practice and the IFRA Standards.



### THE HUMANE SOCIETY OF THE UNITED STATES

The Humane Society of the United States is working tirelessly to decrease and eventually end the use of animals for harmful research and testing. We work toward this goal by focusing on key areas such as eliminating cosmetics testing on animals through our Be Cruelty Free campaign, ending the use of dogs for testing, expanding the development and use of non-animal methods, and ensuring retirement of chimpanzees from laboratories to sanctuaries as soon as possible.



# #UseScienceNotAnimals

Every product Unilever makes must be safe for people to use and safe for our planet. We believe that animal experiments should not be used to make sure that our products are safe.

Unilever started to develop non-animal approaches to assess the safety of its products and ingredients over 40 years ago and we are committed to using what we have learnt to **help accelerate use of new science and technology in chemical and product safety assessment, to ultimately replace the need for animal test data.**

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 ([tt21c.org/resources/](http://tt21c.org/resources/)).

In the spirit of this goal, we would like to welcome you to the 11<sup>th</sup> World Congress on Alternatives to Animal Use in the Life Sciences and invite you to come and meet us at one of our events:

- Oral & poster presentations throughout the Congress
- Unilever - sponsored sessions - live panel discussions & presentations throughout the congress and YOU-WC11 welcome reception
- Congress booth for more information and any questions you may have

Everything Unilever shares during the WC11 is available for download here: <https://tt21c.org/events/WC11/>





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ALTEX Proceedings publishes Abstract Books and Proceedings of scientific conferences and symposia on the development and promotion of alternatives to animal experiments according to the 3R concept of Russell and Burch: Replace, Reduce, and Refine in cooperation with the organizers of the respective meeting.

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