LINZ 2022 – EUSAAT 2022
Volume 10, No. 2
ISSN 2194-0479
(2022)

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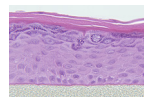

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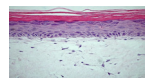

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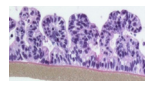

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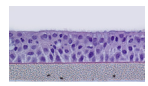

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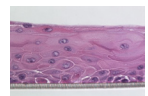

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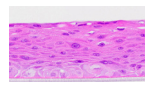

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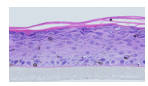

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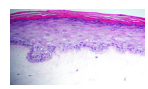

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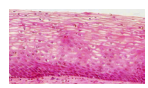

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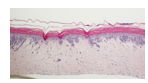

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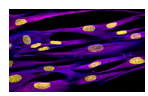

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Dear friends and colleagues,

On behalf of EUSAAT, the European Society for Alternatives to Animal Testing, we welcome you to the 23rd European 3Rs Congress and the 20th EUSAAT Congress in Linz, Austria from September 26-28, 2022.

Since 1991 the "Linz-Congress" has become the main event in the field of 3Rs science in Europe. After two years of the Covid-19 pandemic, which entailed challenging travel restrictions and lockdowns, we are excited to welcome you back to Linz to celebrate the 3Rs and explore avenues for improvement and innovation of animal testing with a 2022 "in-person" conference. Among the many registrations of friends and colleagues, we particularly value the upcoming reports of our international partner, the Japanese Society for Alternatives to Animal Experiments (JSAAE), on their recent activities in our session on "3R Centers in Europe & International".

As usual, EUSAAT 2022 hosts oral and poster presentations to facilitate discussions and exchange ideas to promote the most advanced non-animal methods in the life sciences. The Scientific Committee has put together a stimulating and appealing program on the most exciting and recent subjects that are currently the focus of 3Rs research in Europe and worldwide.

The *main program focus is on Replacement methods as the most critical pillar of 3Rs research*, with sessions on the Covid-19 pandemic and the 3Rs, non-animal models, and novel technologies for skin, eye, lung, and cardiac toxicity, liver, and intestine, cancer research, ecotoxicology, developmental neurotoxicity (DNT), *in silico* toxicology, stem cell models, biological barrier models for infection studies, replacement of animal-derived products, NAMs for risk assessment and human disease models.

As with previous events, the EUSAAT Congress 2022 also constitutes a platform for the other two pillars, *Reduction, and Refinement*, including sessions and topics such as preregistration and best practice, quality aspects and funding, refinement and pain assessment, and 3Rs policy accelerating the transition to innovation without the use of animals.

Based on recent developments in Europe and the great success of previous EUSAAT congresses, we will once again hold several sessions on establishing the *3Rs Centers network in Europe* and addressing their international role in improving education and accelerating active implementation. During the Round Table Discussion, we invite you to reflect on past achievements and assess recent challenges in light of the progress and alignment between the different initiatives.

We are very pleased that distinguished colleagues have followed our call to Linz as keynote speakers to enrich our program with various perspectives and exciting insights on recent achievements and scientific progress. Aside from excellent Austrian cuisine, the Gala dinner will host the award for the best ALTEX article of 2021, and we will sign a Memorandum of Understanding with ESTIV.

Other highlights of this year's conference are the YOU events spread throughout the EUSAAT 2022 conference program, which are aimed at young and early career scientists (up to the age of 35) working or planning to work in the 3Rs field. We want to facilitate the dialogue between young scientists and established mentors to encourage professional networking opportunities and provide a free and open atmosphere for exchanging scientific ideas and career perspectives in the 3Rs field!

EUSAAT continues to support early career scientists with the "Young Scientists Travel Awards (YSTA)" with the help of the SET Foundation's generous funding. The YSTA program provides 20 young scientists with the opportunity to share their ideas on implementing the 3Rs in education and the use of novel test systems to reduce animal suffering and overall numbers in research. AnimalfreeResearch, EPAA and *ecopa* sponsor five additional travel grants, and ATLA supports our youngest with Young Investigator Awards for best conference presentations.

The EUSAAT Board is pleased to announce that the amount of sponsoring of the EUSAAT congresses is continuously increasing. The generous financial support of our sponsors can keep the scientific standard high and the congress fees low. This central EUSAAT ideology attracts young scientists from Europe and beyond. Thus, on behalf of the participants, the EUSAAT Board, and the Scientific Committee, we thank our sponsors. Also, we thank Sonja von Aulock, editor and CEO of ALTEX Edition, for her support to again promote our conference abstracts via publication in ALTEX Proceedings.

Finally, we want to thank our colleagues on the EUSAAT Board, the Scientific Committee, and the Co-Organizers for their continuous support in planning this year's EUSAAT congress.

Winfried Neuhaus	Annemarie Lang	Mario Rothbauer	Horst Spielmann
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List of Abstracts

1

Investigation of ADHD-associated alterations of the neurovascular unit by aid of hiPSC-derived *in vitro* models

Markus Glaser[1], Zora Schickardt[2], Sabrina Oerter[3], Rhiannon McNeill[2], Carolin Koreny[2], Christian Lotz[3], Sarah Kittel-Schneider[2] and Antje Appelt-Menzel[1,3]

[1]University Hospital Würzburg, Chair Tissue Engineering and Regenerative Medicine, Würzburg, Germany; [2]University Hospital Würzburg, Department of Psychiatry, Psychosomatics and Psychotherapy, Würzburg, Germany; [3]Fraunhofer Institute for Silicate Research ISC, Translational Center Regenerative Therapies TLC-RT, Würzburg, Germany

Attention deficit hyperactivity disorder (ADHD) is the most prevalent neurodevelopmental disorder worldwide and one of the most heritable mental disorders. Environmental risk factors such as low birthweight and prematurity are thought to contribute to an ADHD phenotype [1,2]. While molecular mechanism underlying ADHD etiology and stimulant response remain unclear, not only common but also rare genetic variants like copy number variations (CNVs) of common risk genes might play a role in ADHD [2]. CNVs in the PARK2 gene have been shown to be associated with ADHD [3]. Thus, genetic variants and mutations in PARK2 might result in protein dysfunction, thereby impacting mitochondrial stability.

Blood-brain barrier (BBB) disruption has been implicated in ADHD [4], since changes in BBB functionality and metabolic activity can lead to barrier opening and a disrupted microenvironment, impairing neuronal physiology. Taken together, there appears to be a link between ADHD and mitochondrial as well as BBB dysfunction, which has not been investigated previously. Furthermore, treatment with stimulant medication can alleviate symptoms of ADHD, however about 50% of adult patients are non- or only partial-responders to pharmacological therapy [5]. Therefore, the development of novel medication is necessary in order to address human-specific cellular metabolic changes.

The aim of the project is to establish an advanced *in vitro* test system of the neurovascular unit (NVU) combining the BBB with the adjacent central nervous system to recapitulate human-specific ADHD-related cellular changes and facilitate drug screening in ADHD treatment.

Fibroblasts of *PARK2* CNV carriers and healthy controls were reprogrammed in human induced pluripotent stem cell (hiPSC) lines. According to established differentiation protocols, patient-specific hiPSC-derived cortical neurons and brain capillary-like endothelial cells (BCECs) were generated and characterized, respectively. Therefore, specific marker expression, electrical potentials, barrier integrity, mitochondrial biogenesis, morphology and energetics were assessed.

For the development of isogenic NVU models, co-cultures of hiPSC-derived BCECs and neurons were initially generated in static transwell-based setups and subsequently under microfluidic culture conditions. Compared to respective mono-culture, complex crosstalk between cortical neurons and BBB endothelial cells was examined. Additionally, investigations of shear stress-induced effects on the BBB phenotype were performed.

As an alternative to animal studies, the humanized model can be used in future studies to explore the influence of environmental risk factors and to test novel therapeutic approaches. Replication of gene-environment interaction will be realized by simulating ADHD-associated risk factors such as prematurity, hypoxia, inflammation and oxidative stress. In addition, therapeutic approaches like stimulant medication and antioxidants will be validated to restore mitochondrial and cellular function.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

2

Animals in the (Petri) dish: Towards a truly animal-free laboratory

Tilo Weber

Animal Welfare Academy of the German Animal Welfare Federation, Neubiberg, Germany

Directive 2010/63/EU calls for a full replacement of procedures on live animals for scientific and educational purposes as soon as it is scientifically possible to do so. One approach for Novel Approach Methodologies (NAM) is the use of cell and tissue cultures. To facilitate an optimal environment for cells to thrive *in vitro*, they have to be held in homeostasis by maintaining media supplement, attachment factors, coating materials and even preparation of the used plastic ware [1].

Unfortunately, many commonly used laboratory reagents and materials are still of animal origin. This does not only cause immense ethical and animal welfare issues and dilemmas. Moreover, their use can result in reduced reproducibility, reliability, transferability and biosafety of any scientific data derived from these methods. Therefore, animal-derived materials and reagents have to be replaced by human-derived or (chemically) defined materials to fully end the exploitation of animals in science.

In this presentation, different sources of animal-derived laboratory products will be described; alongside with production procedures and the implication for involved animals. Main focus will be on production of fetal bovine/calf serum (FBS/FCS) [2], but also murine sarcoma cells, animal-derived antibodies and pyrogen detection tests. Furthermore, an ethical discussion of the use of non-human-derived cells will be encompassed [1].

Finally, cruelty-free replacements will be highlighted, including strategies to accelerate transition to non-animal-derived laboratory reagents and materials [3]. Thence, setting the path to achieve a truly animal-free laboratory working environment to the benefit of animals, scientists and patients.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

3

Identifying skin sensitizers with an innervated 3D-skin model

Wiebke Bergforth, Maren Schenke and Bettina Seeger

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Substances that come into contact with human skin must be tested for their potential to sensitize the skin before they can be approved for use in products such as cosmetics. The OECD published a guideline on Defined Approaches for Skin Sensitization [1] containing a number of *in vitro*, *in chemico* and *in silico* methods to characterize the skin sensitizing potential of chemicals without the use of animal testing. These methods are based on the original Adverse Outcome Pathway which includes tests for several key events such as the binding to skin proteins, activation of keratinocytes and dendritic cells leading to the final Adverse Outcome: skin sensitization. Within the Defined Approaches, different aspects of these tests allow for both the *identification* of potential skin sensitizers as well as a *potency categorization.*

In this project, the quantification of neurite outgrowth in a three-dimensional skin model is used to develop an assay to model itch *in vitro*, seen in the context of allergic contact dermatitis to complement other *in vitro* assays established in the *in vitro* testing battery and to be expanded for other scientific questions on sensory neuron-mediated chronic pain in the future.

Therefore, a hydrated collagen matrix is seeded with primary fibroblasts and keratinocytes to form a basic epidermis model and is complemented by seeding induced pluripotent stem cell-derived sensory neurons and Schwann cells, based on the protocol of Muller et al. [2]. Some skin sensitizers are able to directly bind to Transient Receptor Potential Vanilloid 1 (TRPV1) on sensory neurons or they could induce Nerve Growth Factor (NGF) production in surrounding cells, like the directly exposed keratinocytes, both leading to neurite outgrowth, associated to itch [3].

Currently the sensory neurons and Schwann cells have successfully been differentiated from the iPSC cell line IMR90-04 and have been characterized using RT-qPCR and immunocytochemistry. Expression of sensory markers Brn3a, TrkA and TRPV1 as well as Schwann cell markers SOX10, S100b, GFAP, APOD, MPZ and PLP1 was verified.

Current work focuses on characterizing the innervated 3D skin model by immunocytochemistry and on analyzing keratinocytes for their potential to produce NGF in response to sensitizing substances. Next steps will be to analyze gene expression (neuron sensitization associated upregulation of ion channels, pain receptors and proinflammatory profile) and neurite outgrowth after application of skin sensitizers and non-sensitizers.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Ethics vote is not necessary.

4

Towards an integrated approach to testing and assessment for cardiotoxicity

Sivakumar Murugadoss[1], Ronette Gehring[2], Tom Roos[2], Nunzia Linzalone[3], Birgit Mertens[1] and Martin Paparella[4]

[1]Sciensano, SD Chemical and Physical Health Risks, Brussels, Belgium; [2]Veterinary Pharmacotherapy and Pharmacy, Department of Population Health Sciences, Utrecht University, Utrecht, The Netherlands; [3]Institute of Clinical Physiology, National Council of Research, Pisa, Italy; [4]Division of Medical Biochemistry, Medical University Innsbruck, Innsbruck, Austria

The heart is one of the vital life-supporting organs. Since the heart has a high energy requirement, receives a high blood flow and is metabolically very active, it can be a vulnerable target site for stressors. Toxic responses of the heart following exposure to various stressors such as drugs, natural compounds and environmental pollutants can cause cardiotoxicity, and contribute to certain heart diseases such as cardiomyopathy and cardiac arrhythmia, ultimately leading to heart failure [1]. However, compared to drugs, potential cardiotoxic mechanisms of environmental pollutants and chemicals are poorly understood. This represents a relevant uncertainty for the regulatory assessment of environmental pollutants and chemicals. Current regulatory hazard assessments are widely based on animal testing, focus on apical toxicity endpoints, and provide only minimal information on the mechanism or mode of action. Hazard assessment of a large number of chemicals by animal testing is also unfavorable as it is expensive, time-consuming and unethical. To reduce the gap between the number of chemicals in use and the number assessed for their hazards to date, there is an urgent need to develop cost-effective and efficient regulatory toxicity testing approaches. Integrated Approaches to Testing and Assessment (IATAs) have been proposed as science-based approaches for chemical hazard characterization. They usually start with an integrated analysis of all existing toxicological information, such as data from computational models, traditional *in vivo, in vitro* and *ex vivo* testing. The outcome of this analysis defines, if and which new approach methodologies (NAMs) shall be applied *as a second step* to complement available data for supporting regulatory decision making [2]. Today, NAMs are not yet widely and consistently used for regulatory decision-making due to a lacking mechanistic understanding of toxicity. The Adverse Outcome Pathway (AOP) framework can be applied to characterize mechanistic relevance of novel methods in predicting an adverse outcome. Thus, AOPs can be used as a framework to develop IATAs [3]. In this work, we identify the current regulatory data requirements for cardiotoxicity outlined in the European Commission (EC) regulations and guidance for industrial chemicals, pharmaceuticals, pesticides, and biocides. Furthermore, we review the available epidemiological and toxicological evidence for cardiotoxic environmental pollutants and chemicals. Meanwhile, we also aim to collect mechanistic insights in cardiotoxicity induced by environmental pollutants and chemicals using the AOP framework. Finally, an IATA for cardiotoxicity will be drafted according to the international accepted paradigm [4]. The outcome is expected to provide the outline of a potential practical regulatory approach to assess the cardiotoxic potential of environmental pollutants and chemicals, taking into account existing data sources, models, and NAMs for cardiotoxicity.

This work was supported by the European Union's Horizon 2020 research and innovation program (grant # 101037090). The content of this manuscript reflects only the author's view, and the Commission is not responsible for any use that may be made of the information it contains. The work of Martin Paparella at the Medical University of Innsbruck is financed by the Austrian Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology, Department V/5 – Chemicals Policy and Biocides.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote

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A novel three-dimensional *in vitro* biofilm model in combination with human tissues – Towards predictive chronic infection modeling

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Constantly increasing incidence of chronic tissue infections constitutes a serious burden on individual patients as well as healthcare systems. 60-80% of such chronic infections are associated with bacterial biofilms, defined as bacterial aggregates surrounded by a self-produced matrix of extracellular polymeric substances (EPS). As this particular lifestyle provides several tolerance mechanisms against antimicrobials and the hosts' immune defense system, the presence of bacterial biofilms significantly contributes to the prolonged and persistent disease characteristic of chronic infections. Against this background, predictive biofilm models are urgently needed. On a large extend, current biofilm infection research is based on animal models, predominantly rats and mice. However, differing anatomies and divergent pathophysiological mechanisms of tissue infections lead to a limited translatability of the results to the human system. Additionally, *in vivo* infection experiments generally result in severe, long-lasting pain for the test animals. This is particularly true for biofilm-related infections due to extended biofilm formation and maturation periods, often associated with high mortality rates. Therefore, a focus must be set on the development of predictive *in vitro* biofilm models. Although several *in vitro* models have been developed, they are mostly characterized by an artificial, two-dimensional growth environment and a lack of living host cells, limiting their potential for *in vivo* predictability. To overcome this limitation, we developed an *in vitro* biofilm model based on electrospun fiber mats. The three-dimensional polymeric scaffold of electrospun fibers allows for precise imitation of the extracellular matrix of bacterial biofilms while providing a mechanical support for the model. Thereby, the transfer of mature biofilms to human tissue models was enabled, receiving an *in vitro* chronic infection model.

Based on the composition and structure of native EPS, gelatin in combination with cellulose acetate were selected as suitable biocompatible polymers to be processed via electrospinning. This technique allows to produce three-dimensional polymeric fiber networks with fiber diameters in the nanometer size range. The obtained fiber mats were analyzed by multiple analytical methods such as contact angle measurements, tensile testing, and scanning electron microscopy (SEM). Required physicochemical properties of the scaffold included uniform fiber morphology, suitable fiber diameter, moderate hydrophilicity, and high mechanical strength. After a characterization and optimization procedure, the most promising fiber mats were used as scaffolds for biofilm cultivation.

Electrospun fiber mats were inoculated with *Pseudomonas aeruginosa*, a common opportunistic pathogen of human tissue infections. Subsequently, the biofilm models were incubated for 48 hours on modified nutrient agar plates. Quantification of colony forming units revealed a natural growth behavior of the bacteria. A homogenous bacterial distribution throughout the entire fibrous scaffold further confirmed the polymeric fibers as an appropriate substrate for bacterial growth. The successful maturation of biofilms was demonstrated by visualizing EPS via histological staining of cross sections as well as SEM. As an enhanced antimicrobial tolerance compared to the planktonic phenotype is a main property of native bacterial biofilms, susceptibility testing of the developed *in vitro* biofilm model was performed. Using gentamicin as a model antibiotic, a highly reduced susceptibility of the biofilm model compared to planktonic *Pseudomonas aeruginosa* was shown, highlighting the similarity to the *in vivo* situation in biofilm-infected tissues.

In a next step, pre-grown mature biofilm models were transferred to *ex vivo* human skin wounds, resulting in a continuing viability of the biofilm model. Furthermore, histological investigations and SEM confirmed a close contact of the biofilm model to the skin tissue. Bacterial colonization of the wound bed and beginning invasion in the skin was observed after 24 hours of co-cultivation.

To conclude, we successfully developed an *in vitro* biofilm model, which served as a versatile platform model for simulating chronic human tissue infections in a clinically relevant way. By precisely imitating the microenvironment of biofilm-related infections and providing typical characteristics of *in vivo* biofilms, the developed model poses a valuable alternative to routinely used animal models. In the future, the complexity of the model can be increased by extending it to multiple species in combination with *ex vivo* as well as *in vitro* tissues.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: The study was registered with the local ethics committee and informed consent of patients was received.

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Generation of reporter models for rapid oxidative stress induction measurement *in vitro* and application to 3D bronchial tissue cultures

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The development of novel three-dimensional (3D) reporter models derived from human primary airway cells can facilitate the *in vitro* toxicological assessment of whole aerosol in close-to-physiological conditions. Here, we used genome engineering to generate reporter cells which can be used to conveniently monitor the induction of cellular stress upon exposure to test aerosols.

We chose CRISPR-Cas9 technology to tag the oxidative stress marker heme oxygenase 1 (HMOX1) with green fluorescent protein (GFP). We successfully established this method in the HEK293 human embryonic kidney cell line, in which five different guide RNAs (gRNAs) were screened. One particular gRNA allowed the efficient insertion of the *GFP* close to the stop codon of *HMOX1*, which led to the expression of C-terminal HMOX1-GFP fusion protein. The induction of the fusion protein was driven by the endogenous promotor of *HMOX1* and could be assessed easily by measuring fluorescence intensity. We applied this method to the BEAS-2B human bronchial epithelial cell line and verified the expression of the fusion protein by western blot and fluorescence microscopy. In these reporter models, HMOX1-GFP was induced in a dose-dependent manner upon treatment not only with the oxidative stress inducer hemin but also with cigarette smoke fractions.

To generate more physiologically relevant reporter models, we used a similar approach with normal human bronchial epithelial (NHBE) cells. These cells can be cultured and differentiated at the air-liquid interface to generate 3D bronchial organotypic tissues. Our genome engineering strategy required adjustments due to the inherent low transfectability and low proliferation rate of primary cells. We delivered CRISPR-Cas9, gRNA and the DNA repair template to HEK293 and BEAS-2B cells in the form of plasmids by electroporation. For NHBE cells, we pre-assembled the recombinant CRISPR-Cas9 protein with the *in vitro*-transcribed gRNA and used electroporation to transfer this ribonucleoprotein complex into the cells. The DNA repair template was packaged into adeno-associated virus particles, with which the electroporated cells were subsequently infected. Preliminary results indicate that NHBE cells can be successfully transfected and genome-edited. As a next step, we will assess if the modified cells can be used to generate bronchial tissues which can be exposed directly to whole aerosols.

Submission declaration:
Conflicts of interest: Philip Morris International is the sole source of funding and sponsor of this research.
Statement on ethics vote: There was no ethics vote needed.

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Use of organotypic gingival cultures for the assessment of nicotine pouches

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Nicotine pouches (NP) are an emerging class of nicotine products for oral use. The appearance and usage of NP are similar to those of the Swedish snus, but they do not contain tobacco. They consist of a nicotine-containing cellulose matrix inside a fiber sachet, with flavor-imparting and other ingredients, such as fillers, stabilizers, pH adjusters, and noncaloric sweeteners. NP with different nicotine strengths and flavors are available in different markets worldwide. Owing to the absence of tobacco and combustion, NP are considered by the manufacturers to pose a lower risk to consumer health than smokeless and combusted tobacco products. However, few studies have investigated the biological effects of NP.

In this study, we used human organotypic gingival epithelial cultures to assess the biological effects of NP. We optimized a methodology to extract NP in an aqueous solution in a reproducible and controlled manner. We also assessed nicotine yield and stability of the key components of the NP extract over time. Organotypic gingival cultures were apically exposed to the extracts obtained from five types of NP (with different nicotine and flavor contents) and one snus product (two nicotine concentrations: one relevant to consumer use and a higher concentration) for 96 h. Liquid fractions from cigarette smoke (total particulate matter combined with the gas vapor phase) were also tested. At the end of the exposure period, we collected the basolateral medium for the analysis of a panel of 12 secreted inflammatory mediators using Luminex technology, and the morphology of the exposed organotypic tissue cultures was evaluated by a histopathologist after hematoxylin and eosin staining.

We observed that the extracts from two types of NP and the snus had a similar mild impact on the morphology of the gingival cultures at nicotine concentrations relevant to human exposure. The extract from the NP containing a high level of menthol induced a more pronounced effect on gingival morphology, whereas the NP with the lowest nicotine content had the lowest effect. A similar product-dependent effect was observed in case of inflammatory mediator profiling, wherein the high-menthol NP extract was observed to induce the strongest inflammatory response. Exposure to the cigarette smoke fraction had the maximum impact on culture morphology and inflammatory response, even at nicotine concentrations 40 times lower than those of the oral products.

Therefore, the biological effects of NP may depend on their formulation. The impact of NP and snus extracts on gingival organotypic cultures was lower than that of the cigarette smoke fractions, even at higher nicotine concentrations. The use of the human organotypic gingival model, in addition to being compliant with the reduce-replace-refine (3R) approach to reduce the use of animals in research, proved to be a suitable system to differentiate the effects of exposure to NP, snus, and cigarette smoke fractions.

Submission declaration:
Conflicts of interest: Philip Morris International is the sole source of funding and sponsor of this research.
Statement on ethics vote: No ethic vote needed.

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Establishment of a human synovium-on-a-chip for osteoarthritis disease modeling using animal-free technologies

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Shifting away from cartilage-focused research in knee osteoarthritis (kOA) to tissue-tissue communication, the contribution of fibroblast-like-synoviocytes (FLS) to the pro-inflammatory microenvironment is of uttermost importance and should be considered more precisely in the aspect of a whole-joint disease model. This project aims to establish a 3D biochip model of human synovium fulfilling the unmet need for disease-authentic recapitulating *in vitro* models withholding sufficient predictive power as possible drug screening tools. With the incentive of replacing the current state-of-the-art animal-derived products for synovial *in vitro* modeling and increasing human relevance, patient-derived FLS cultures of kOA origin were applied and 3D culture conditions were replaced with human-derived products. Cellular metabolic activity of 2D and 3D FLS cultures with human-platelet-lysate as serum surrogate was quantified via esterase activity and the effect of different hydrogel compositions on construct stability and tissue morphology was elaborated by microscopy. Deriving tissue constructs were benchmarked against synovial kOA patient tissues followed by qPCR applied to monitor the general baseline expression of characteristic synovial physiology as well as kOA marker genes and proteins in synovial organoids. With retaining the highest degree of architectural *in vivo* morphology and patient heterogeneity animal-derived products could be replaced. Constructs deriving from synovial monocultures displayed synovial pathologies such as lining-layer hyperplasia, matrix-component overexpression, and matrix degradation. Synovial physiology indicators (COL1A1, COL3A1, PRG4) additionally to kOA marker (IL6, IL8, MMPs, ADAMTS5) mRNA-expression analysis served to analyze the baseline values as well as cytokine reactivity of the optimized animal-product-free synovium-on-a-chip model. The current work successfully demonstrates the development of an animal-free osteoarthritic synovium-on-a-chip model to recapitulate molecular, cellular, and tissue-level kOA pathologies.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

9

Bioengineering the inflammatory and degradative cross talk environment in arthritic diseases with patient-derived heterotypic joint-on-a-chip systems

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Arthritic musculoskeletal diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) concern great parts of our global population. Tissue communication during onset and progression is characterized by inflammatory as well as degradative molecular processes that in the case of severe pathologies often end in severe surgical interventions. The inflammatory and degradative interaction of joint tissues plays a vital role to aggravate inflammation, matrix remodeling and tissue degradation in both arthritic diseases. As the exact tissue contribution in the complex arthritic environment is mostly elusive, the present study aims to investigate individual tissue contribution to the overall joint tissue crosstalk in arthritic diseases using joint-on-a-chip technologies.

Primary human RA and OA patient-derived synovial-like fibroblasts and articular chondrocytes from OA origin were isolated enzymatically from fresh tissue specimens that in turn were also prepared for histology and qPCR analysis. Initiation of joint-on-a-chip mono- as well as co-cultures were performed by injecting 50 µL aliquots of freshly isolated synovial and chondral cells mixed with 50 mg/mL TISSEEL fibrin hydrogel within individual on-chip tissue compartments at a final concentration of 2,000 and 3,000 cells per µL, respectively. Mono- and co-culture were maintained up to four weeks in the presence and absence of chondrogenic growth factors and analyzed for mRNA expression and protein secretion of (patho-)physiological mediators, proinflammatory cytokines and matrix proteases. Moreover, histological as well as time-lapse imaging were employed to elaborate on tissue-architectural and cell morphology changes over a cultivation period of up to four weeks. A synovial biochip kOA marker expression baseline on gene- and protein level was successfully established.

Three-dimensional on-chip cultures of primary patient-derived cells retained tissue-like characteristics and *in situ* morphologies for both cartilage as well as synovial tissue-like constructs. Analyzing a broad palette of functional checkpoint parameters ranging from tissue architecture, cell morphology, protein secretion profile as well as mRNA expression, we observed that independent of tissue origin, fibroblast-like synoviocytes promoted a more chondrogenic microenvironment in chondro-synovial co-cultures compared to the respective chondral monocultures. While chondral and synovial monocultures were reactive to inflammatory cytokines including tumor necrosis factor TNF-α and interleukin IL-1β and as expected escalated an arthritic environment with upregulation of inflammatory mediators (IL-6, IL-8) and matrix metalloproteinases (MMP-1, MMP-3, and MMP-13), the presence of fibroblast-like synoviocytes as synovial organoid weakened the overall inflammatory mRNA expression response to proinflammatory cytokines. Interestingly, other investigations on chondro-synovial crosstalk for a variety of medium supplementations including chondrogenic growth medium and TGFβ supplementation hampered the formation and maturation of synovial organoids.

To conclude, organs-on-a-chip can provide a technology platform to generate new insights into molecular and biophysical cues at the cellular microenvironment that may govern onset and progression of arthritic diseases such as OA and RA *in vitro* at high precision and reproducibility. Although osteoarthritis in particular can still be considered a cartilage disease by its research focus, the observation of tissue communication as in our current study shows the very promising findings to better understand the complex microenvironment of a plethora of tissues and individual cell populations that govern the inflammatory and degradative disease onset and progression.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Quality enhancing measures in organ model research

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Organ models are promising new approaches providing unique opportunities for the study of human diseases and treatment designs. However, proving the reliability and robustness of these novel approaches will be crucial for their broad acceptance and successful application in translational biomedical research. The *BIH QUEST Center for Responsible Research* is to establish quality standards in organ model research, based on data integrity principles and focusing on scientific value.

Here, we report on quality measures currently being established at the *Einstein Center 3R*, a recently founded, Berlin-based consortium of organ model experts. We are introducing a modular quality assurance system specifically tailored to *in vitro* research in academia. We are building up our approach on recent experiences as a quality management team in another academic research consortium in 2020/21 [1]. Additionally, we are leading a large group of model experts conducting a systematic review assessing the contribution of organ model systems to COVID-19 research. The systematic review focuses on the scientific outcome of infection studies on human organ models, but also assesses the quality of reporting in organ model research [2].

We closely collaborate with researchers in the *Einstein Center 3R* to increase the internal and external validity of their organ model systems and map out different dissemination strategies to laboratories who want to take up the technology (Replace/Reduce). By analyzing the transferability of organ models, we evaluate challenges and define boundary conditions. To increase methodological rigor and transparency we are initiating protocol standardization and publication, including the development of minimum reporting standards in organ model research.

Quality measures in an academic research environment have to be pragmatic and science-driven and allow for innovation, ensure data integrity, and an economic use of resources. This approach has to evolve over time and need the continuous contribution and willingness from laboratory-based scientists, as well as a patient but persistent QM team.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote was required.

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A human *in vitro* model for airway epithelial injury and regeneration

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The human airway epithelium is the primary target for inhaled toxicants and plays a key role in the development of environmentally triggered chronic lung diseases. To date, our knowledge of airway regeneration mechanisms largely stems from mouse models where exposition, e.g., polidocanol (PDOC) causes depletion of bronchial epithelial cells followed by regeneration. Such acute injury to the airway epithelium is associated with substantial distress and suffering for the animals. Furthermore, the cellular composition of the upper respiratory epithelium differs considerably between mice and humans. Therefore, we set out to develop a human-derived *in vitro* model for the study of airway injury and regeneration. Primary human bronchial epithelial cells (phBECs) were differentiated at the air-liquid interface, treated with PDOC, and cell loss and regeneration were monitored by immunofluorescent (IF) staining, qRT-PCR, and transepithelial electrical resistance over four weeks. Treatment with 0.04% polidocanol led to initial cell depletion and subsequent regeneration, characterized by the emergence of all major differentiated cell types at the expense of basal cells and the reestablishment of barrier integrity. Inhibition of Notch signaling by the ɣ-secretase inhibitor DAPT during the regeneration phase abolished differentiation towards secretory cell types, serving as a proof-of-concept that the model is suitable to study mechanisms of airway epithelial injury and repair. In conclusion, PDOC treatment of phBECs *in vitro* allows for the elucidation of mechanisms involved in human airway repair and regeneration and therefore represents a suitable alternative to animal models of airway injury and regeneration.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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RE-Place: Stimulating knowledge sharing on new approach methodologies via an open access database in Belgium

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Although the 3Rs Principle (Replacement, Reduction, Refinement) was already introduced more than 6 decades ago, the replacement of animal testing remains a challenge in the 21st century. New and innovative technologies and methods can help to reduce or sometimes even avoid the use of experimental animals in the life sciences. They are referred to as "New Approach Methodologies (NAMs)" and include, amongst others, computer modeling, high throughput testing methods, the use of -omics, and sophisticated cell- and tissue cultures. Even though the importance of NAMs is gaining interest worldwide, their international acceptance and (regulatory) implementation is often lagging behind.

Stimulating the development and use of NAMs may benefit from a bottom-up approach, i.e., local initiatives mapping the available expertise on NAMs and promoting their use. An example of such an initiative is the RE-Place project in Belgium, funded by the Flemish and Brussels government and coordinated by Sciensano and Vrije Universiteit Brussel (VUB). The goal of RE-Place is to collect the available expertise on NAMs in one central open access database. Due to the fast development pace of NAMs, (young) scientists may have difficulties to find relevant, reliable, and up-to-date 3R-information in an efficient way. The RE-Place database facilitates the search for this type of information. Moreover, the database is unique in the fact that existing NAMs are linked with the names of experts and local research centers where the technologies are currently developed and/or applied. This type of information is often not readily available to all stakeholders involved (scientific community, ethical committees, regulators, the government, etc.). By having a direct point of contact, networking activities are strongly encouraged and facilitated, allowing to speed-up the development and validation processes which are needed for the actual implementation of NAMs in routine practices.

The RE-Place team developed a template to collect the information of interest in a fast and consistent manner. To simplify the information collection procedure, a web-based application was set-up that allows to directly integrate the submitted data into the open access database. Aspects such as user-friendliness, incentives for collaboration, quality check of submitted information, protection of personal data and Intellectual Property (IP) rights were all considered during the development process. By consulting the database, available via www.RE-Place.be, scientists from various research disciplines and regulatory domains can easily identify the different types of NAMs and the experts using them in Belgium. In May 2022, the RE-Place database contained 191 methods submitted by 131 experts from 23 different (research) institutes. The majority of the submitted methods is situated in basic and applied research and is categorized as an *in vitro* or *ex vivo* method.

In addition to actively collecting the existing expertise on NAMs in Belgium, the RE-Place project also promotes the use of new technologies by sharing (inter-)nationally available knowledge via the website and corresponding social media channels. Face-to-Face meetings are organized with experts from various life science areas to present the project and highlight the importance of NAMs, ensuring a close and reliable interaction with all involved stakeholders. As such, the RE-Place project not only helps to raise awareness, but also builds bridges and increases trust in the use of these new technologies, thereby stimulating the regulatory uptake of NAMs and their daily use.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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

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The United States and PETA Science Consortium International e.V. are leading an investigation into the use and analysis of control fish in toxicity studies. The aim of the project is to investigate whether it is possible to use only a solvent control and omit a water control when a solvent is required in fish chronic toxicity tests. The use of only one control would substantially reduce the number of fish used in tests when solvents are required and also save on the resources required to conduct such studies.

A fish early-life stage (FELS) toxicity control database including both solvent and water controls was developed to determine whether for one or more required responses, there are systematic differences between the two controls. For each response, the distributions of control data (means, between- and within-replicate variances) for water, solvent, and pooled controls were investigated. In addition, collected FELS concentration-response data using several fish species and the widely used solvent dimethylformamide are used to investigate the treatment effect (NOEC, ECx regressions) and concentration-response curves are developed when using the water, solvent or pooled controls to give side-by-side comparison of results (NOEC or ECx) using the three choices for control(s). Computer simulations to determine the potential impact on ECx estimation and NOEC values of using only the solvent control in FELS toxicity studies covered the observed ranges of variability (including heterogeneity or overdispersion) and concentration-response shapes in each type of response across species.

The study explores all response variables required in FELS toxicity studies including hatching success, time to hatch, time to swim-up, larval survival (intermediate and total), abnormalities, fish length and fish weight at study end. Furthermore, the study explores both model selection and model averaging for ECx estimation, including standard normality-based models and generalized non-linear mixed models. NOEC values are obtained from a decision rule that uses standard normality-based hypothesis tests (Williams and Dunnett, Cochran-Armitage), non-parametric methods (step-down Jonckheere-Terpstra and Dunn) or generalized linear mixed models as appropriate.

The computer simulations showed little impact of eliminating the water control on statistical power or sensitivity, such that there is no systematic effect on NOEC and EC10/20 estimates from using the solvent control compared to combined controls. Findings of the analysis of FELS concentration-response data are also presented.

Submission declaration:
Conflicts of interest: The authors declare no conflict of interest.
Statement on ethics vote: There is a positive ethics vote.

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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 consumed chemicals in direct or indirect contact. 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 [1]. 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 [2]. 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 6 times over 11 days. The transendothelial or transepithelial electrical resistance at the frequency of 1000 Hz ($TEER_{1000}$ Hz) decreased to below 10% in undiluted category 1 chemicals, indicating severe eye damage. However, with the dilution the TEER values increased above 80% of the control indicating a non-irritant and a dose dependent effect. These findings were supported by morphologic analysis of H&E staining in the tissues, displaying dose dependent damage.

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.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The Directive 2010/63/EU is implemented in the Czech Republic in the Act No. 359/2012, amending Act No. 246/1992, on the protection of animals against cruelty. The harmonized legislation covers the protection of animals used for scientific purposes and for toxicological testing within the principles of 3Rs, i.e., reduction of animals in tests, limitation of their suffering by refinement of procedures and replacement of animal testing by alternative toxicological methods. The 3Rs Centre Czech Republic is a public establishment seated at a governmental institution, i.e., the National Institute of Public Health in Prague (NIPH). The 3Rs Centre Czech Republic is actively promoting the 3Rs principles and contributing to the fulfillment of 3Rs strategy. Currently, it functions as a multidisciplinary network comprising National Reference Centres (NRC), National Reference Laboratories (NRL) and the Unit for Alternative Toxicological Methods at the Centre of Toxicology and Health Safety, at NIPH. Thanks to the active multidisciplinary approach, advanced technologies and equipment, the 3Rs Centre Czech Republic covers all three principles of the 3Rs strategy. To cover the Replacement principle, the NRL for Experimental Immunotoxicology focuses on scientific research, development, validation and standardization of New Approach Methodologies (NAMs), such as *in vitro* methods based on 3D tissues and cell lines. The Unit for Alternative Toxicological Methods implements validated NAMs and provides professional testing of chemicals, cosmetics, consumer products and medical devices in compliance with ISO standards, accreditation and GLP quality systems. The dissemination of the 3Rs concept is realized by active participation at national and international events, organized by, e.g., EUSAAT, ESTIV, SETOX (Slovak Toxicology Society), PROKOS (Association of producers, importers and distributors of cosmetics and their ingredients), CLASA (Czech Laboratory Animal Science Association), etc. Members of the 3Rs Centre Czech Republic are involved in international societies such as EUSAAT, ESTIV, serve as experts for OECD, ECHA and EURL-ECVAM. Members of the 3Rs Centre Czech Republic participate in authorization of experimental projects in cooperation with the animal welfare committee at the Ministry of Health, ensuring that the approved projects fulfill the 3R principles required by the Directive 2010/63/EU. With regard to scientific quality/translatability, the Ministry of Agriculture of the Czech Republic has nominated NIPH as the contact point to provide advice on the regulatory relevance and suitability of alternative approaches proposed for validation (PARERE) and the National Reference Laboratory for Experimental Immunotoxicology as a specialized laboratory to the network of qualified laboratories participating in validation of NAMs (EU-NETVAL). The presented poster summarizes the range of activities of the 3Rs Centre Czech Republic.

Supported from ERDF/ESF project “International competitiveness of NIPH in research, development and education in alternative toxicological methods” (No. CZ.02.1.01/0.0/0.0/16_019/0000860) and by Ministry of Health, Czech Republic – conceptual development of research organisation (“National Institute of Public Health – NIPH, IN: 75010330”).

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Transforming culture: Culture of care in animal research

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Introduction and aim: "Culture of Care" is understood as an appropriate behavior and attitude towards all animals and employees. All levels of an institution (management, science, care) are committed by personal responsibility and a proactive attitude. The desideratum becomes particularly clear in the analysis of all levels related to a differentiated meaning of a Culture of Care in animal research.

Materials and methods: Following on from the intensive and differentiated derivation of the historical anchoring and reception in the concept of a Culture of Care, the project focuses on analyzing the characteristics of a Culture of Care in Germany. By using qualitative social research, the management level, the science level, the monitoring level and the care level were analyzed. Three interviews were conducted per level.

Results: Results show that individual knowledge of the concept of a Culture of Care derives from the decisions of an actor in the level and from the given legal and organizational structures. Subsequently, Culture of Care is understood as a complex mosaic of different categories: Organizational level, personality, science and animal welfare are main categories. These categories are underpinned by subcategories such as 3R, ethical concerns, education or agency.

Conclusion: Results indicated that a "simple commitment" is not enough to implement sustainable transformation in the culture of laboratory animal science. A Culture of Care can only be implemented if all levels involved break down old structures and rules and interact with each other. As a result, Culture (of care) is a basis for all research concerning animals.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

17

Cilgavimab/Tixagevimab as alternative therapeutic approach for BA.2 infections

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The identification of the SARS-CoV-2 Omicron variants BA.1 and BA.2 immediately raised concerns about the efficacy of currently used monoclonal antibody therapies. Here, we analyzed the activity of Sotrovimab and Regdanvimab, which are used in clinics for treatment of moderate to severe SARS-CoV-2 infections, and Cilgavimab/Tixagevimab, which are approved for prophylactic use, against BA.1 and BA.2 in a 3D model of primary human bronchial epithelial cells. We observed that Sotrovimab, but not Regdanvimab, is active against BA.1; however, both antibodies lose their efficacy against BA.2. In contrast, we found that BA.2 was sensitive to neutralization by the approved prophylactic administration and the therapeutic use, which is not yet permitted, of Cilgavimab/Tixagevimab. Importantly, while the use of Tixagevimab/Cilgavimab is effective in controlling BA.2 but not BA.1 infection, monoclonal antibodies with efficacy against BA.1 are ineffective to reduce BA.2 virus replication in a human lung model. Our data may have implications on the variant specific clinical use of monoclonal antibodies.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

18

Improving Member State reporting on the implementation of Directive 2010/63/EU

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One of the main objectives of Directive 2010/63/EU ("the Directive") is to reduce disparities, enhance transparency, and create a greater harmonization between and within its Member States regarding the protection of animals used for scientific purposes [1]. However, several studies have reported disconcerting inconsistencies, insufficient and inadequate implementation of the requirements laid down in the Directive, as well as a lack of transparency [2-4]. This heterogeneity in the implementation of the Directive across Member States, mainly because of differences in resources and experience, continues to negatively affect the objective of creating a level playing field for the research community and industry across the EU.

In line with Article 54(1) of the Directive, Member States are required to submit to the European Commission information on its implementation once every 5 years. Reporting on aspects of the implementation of the Directive offers an opportunity to get a comprehensive understanding of how the Directive is implemented within each Member State by assessing the different governance, infrastructures, processes and operations at national level. In 2018, the first information on the implementation of the Directive, covering the first five years of its functioning, i.e., the period 2013-2017, were submitted by all Member States using a EU Survey. An in-depth analysis of this information identified several shortfalls with regard to the understanding of data requirements and to content quality. Shortfalls included unanswered mandatory questions, conflicting information, as well as incomplete or incorrect data, most often because of a misunderstanding of the reporting requirements. However, some of the Member States provided detailed information to mandatory and voluntary questions, as well as additional information to provide a more comprehensive context to the answers or to clarify any concerns.

The analysis of the first information on the implementation of the Directive allowed us to discern how and in which areas Member States can improve their reporting for the second submission of information on the implementation of the Directive, covering the years 2018-2022. To help the Member States improve the quality of their next reports, harmonize data content, and address any inconsistencies, we prepared tailored summary reports for the 14 Member States that use the highest number of animals for scientific purposes and a general report for all other Member States on the basis of the information submitted by the countries to the 2018 EU Survey. The tailored summary reports list the elements that were adequately and not adequately reported by the Member States to help understand where difficulties were encountered, and where more accurate and harmonized information can be provided. Accordingly, the reports provide recommendations on legally required data to be reported as laid out in Annex II of Commission Implementing Decision 2020/569/EU [5], and on how Member States can better report information based on best practices among the answers of the countries to the EC 2018 survey.

Accurate, comprehensive and harmonized reporting of information on the implementation of the Directive can increase transparency on the use of animals for scientific purposes, help Member States learn from each other, as well as unify and optimize aspects of the implementation of the Directive to better progress the application of the principles of the 3Rs.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

19

No improvement in 60 years: Drug failure rates from the 1960s to the 2010s

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Drug efficacy, safety, bioavailability, and other pivotal drug properties are routinely assessed in animals throughout preclinical trials. Internationally, the evaluation of multiple nonclinical drug parameters in living animals is either required by law or regarded as best practice and encouraged by the responsible regulatory agencies like the US Food and Drug Administration (FDA) and the European Medicine Agency (EMA). Simultaneously, the attrition rates during clinical drug assessment have been notoriously high, raising the question of whether current animal-intensive practices are adequate to test drugs for human use.

Here, we aimed to shed light on the development of new drug candidates' clinical failure rates in the last six decades. For this purpose, we analyzed the average attrition rates of drug candidates entering clinical Phase I from the 1960s to the end of 2010s. We investigated data reported in 19 studies for 52 partially overlapping periods and show that in the last 20 years the average drug failure rate exceeds 90%, comparable with the levels in the 1960s. Next, we analyzed the reasons for drug failure in one or several phases of clinical trials from 12 reports. Our results reveal that biological reasons such as safety and efficacy issues, classically assessed in animal trials prior throughout nonclinical drug evaluation, constitute approximately 75% of causes for drug attrition. Taken together, our analysis provides a detailed overview of the drug failure trends in the last 60 years. The fact that drug failure has remained at its highest point in the past 20 years despite the overall scientific and technological advances strongly suggests that the current animal-based preclinical assessment systems cannot be used as reliable predictors of human biology. Our findings indicate that more predictive, human-relevant systems are urgently needed in order to improve preclinical predictability and success rates in drug development.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote is necessary for this report.

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Optimizing animal-free 3D lung model for SARS-CoV-2 research and drug testing

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To study host pathogen interactions in a more physiological manner, that is additionally as close as possible to the *in vivo* situation, the need for improved 3D models is given. During the COVID-19 pandemics once again the importance of human *in vitro* models was highlighted, since lately developed virus variants had to be examined as fast and safe as possible. Especially in such research questions it is essential to work in models that represent the human body in its high complexity. Furthermore, such human 3D *in vitro* models offer a very good base for drug testing, which for example were performed during our studies within a respiratory epithelial 3D model. For this, different mouth and nose sprays were sprayed to the apical side of the lung epithelium prior to infection with SARS-CoV-2. Samples were analyzed by performing TEER measurements and immunofluorescence as well as PCR of subnatants.

Another advantage by working with human derived models is the possible setup of personalized studies, since such *in vitro* models can be designed from patient derived material. Besides these aspects, the optimization of *in vitro* models can help decreasing animal related products or animal experiments in research.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

21

A barrier-on-chip model with integrated 3D stretching to support preclinical decision making

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Introduction: Despite the urgent need for new and better drugs to treat patients, bringing a new drug into the market is a very long, complex, and expensive process. Current 2D *in-vitro* models for drug testing in the laboratory are just too simple to accurately model the drug interaction in the human body. On the other hand, in addition to being ethically questionable, animal models often fail to predict human responses. Therefore, new advanced *in vitro* models such as the AlveoliX Barrier-on-Chip System is needed to better assess the safety and efficacy of drug candidates.

In the present work, we introduce the ^{AX}Barrier-on-Chip System that allows the creation of dynamic environments. This enables to model biological barriers on-chip reproducing *in-vivo*-like conditions of human organs. It consists of two chips closed in a plate (AX12) which provides 12 individual wells, 2 electro-pneumatic devices (^{AX}Exchanger and ^{AX}Actuator), and one docking station (^{AX}Dock) which is the interface between the plate and the two devices. The ^{AX}Exchanger opens valves inside the AX12 allowing to exchange the medium. The ^{AX}Actuator controls the 3D stretching. In the AX12, the cell barrier can be recreated by mono-, co- or multi-cultures. To do so human cells are cultured on each side of the ultrathin porous membrane, which can then be stimulated by 3D motions, recapitulating the dynamics of human organ barriers.

Results: The ^{AX}Barrier-on-Chip System enables to create multiple organ barriers while enabling to perform a wide range of readouts such as TER measurements to assess the barrier integrity, permeability assays to estimate molecule transport across the barrier, proteomics (ELISA) to assimilate secreted biomarkers or immune-mediated cytokine storm, and single-cell transcriptomics to estimate accuracy within vivo populations.

Using human primary lung and human gut cells we successfully created lung and gut barriers on-chip. In both barrier models, the reproducible increase in TER revealed a robust capacity for creating tight and stable barriers on-chip. Furthermore, the presence or addition of 3D movement influences cell differentiation, seen in the expression of the lung- and gut-specific markers. Further, morphological differences were observed after the inclusion of the stretching motions.

Conclusion and discussion: Overall, we have established two advanced barriers on-chip models capable of replicating *in vivo*-like dynamics, barrier formation, and cell-specific markers using the ^{AX}Barrier-on-Chip System. The results show that this tool is suitable for accurately modeling different tissue barriers including *in-vivo*-like mechanical simulation. Such advanced *in-vitro* models have the potential to detect unsafe drugs early in the drug development phase, helping for faster decision making and reducing or even replacing animal experiments. In addition, they enable to create disease models as well as personalized medicine applications.

Submission declaration:
Conflicts of interest: The corresponding author is employed by AlveoliX AG.
Statement on ethics vote: There is a positive ethics vote.

22

Implementation of the 3Rs program in the Comparative Medicine and Bioimage Centre of Catalonia

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The IGTP is a public research center dedicated to increasing scientific knowledge and promoting its transfer to improve the treatment, care and lives of patients. The Institute is attached to one of the large teaching hospitals in the Barcelona area; the Germans Trias i Pujol University Hospital, from the Autonomous University of Barcelona.

In 2019, the Comparative Medicine and Bioimage Centre (CMCiB) was inaugurated [1]. It is a unique 4500 m² new research and training facility in Europe. The CMCiB is a complex multidisciplinary facility designed to meet the needs of comparative medical research and it is devoted to applying the 3R principle and promoting the use of alternative methods for preclinical research. Studies being carried out in the center include an extensive range of research projects, from pre-clinical to clinical using organoids, fly, fish, small and large animal models combined with bioimage, *in silico* and mathematical models. The in-house CMCiB staff include experts in the areas of animal welfare and husbandry, study design, bio contention, surgery, quality, and image diagnostic, bioimage as well as computational analysis methods and mathematical modelling. The CMCiB is a publicly funded research facility that works in collaboration with leading players in the private sector to improve and refine research methods and drive technological innovation for the benefit of society. The 3Rs program is funded by "Fundació La Caixa". The Zebrafish platform is from ZeClinics. Image clinical specialists are from Canon Medical Systems. The mathematical models included in the program are coordinated by Polytechnic University of Catalonia (UPC).

The 3R program is focused on the training and education of the researchers, the promotion of alternative methods, as well as the reduction of the animals used in research. *Drosophila* and the zebrafish are the alternative methods promoted. The use of bioimage techniques and mathematical models allow the reduction of the animals used. The program is financed for 5 years by a private institution, Fundació La Caixa.

To promote that the research groups implement new approaches, the program finances the costs of including them into the design of the research projects. On the other hand, the costs of the staff that work directly on laboratory animal welfare is also covered as well as the investment of bioimage equipment.

After 3 years of activity, the 3R program is fully implemented and some scientific results are arising. Some of the examples are the following. The development of an *in silico* model for the study of tuberculosis disease, The refinement of the electroporation techniques for immunology studies or the reduction of mice used in a pharmacology test thanks to the use of bioimage combined with IA techniques.

In conclusion, the 3R program not only has promoted the replacement, reduction, and refinement of the use of animals in biomedical research, but also has improved the quality of the research and has also promoted the culture of care of the research community.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

23

Role of non-animal technologies in COVID-19 research

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A key characteristic of the SARS-CoV-2 coronavirus is that it can infect and damage multiple human organs such as the lungs, heart, brain, kidneys, intestines, and others. Currently, no other animal species is known to develop the multitude of these complex symptoms or the severe COVID-19 disease observed in critically ill patients. Hence, human-based non-animal technologies (NATs) have been instrumental for the research of the coronavirus' biology, physiology, and infection mechanisms as well as for the development of safe and effective drugs, vaccines, and therapies. Here, we present some examples of the many innovative NATs that play critical roles in COVID-19 research. Potential drug candidates are tested on human lung and alveolar organoids as they can be infected with SARS-CoV-2 via the same molecular mechanisms seen in native human lungs. The first cellular evidence that the coronavirus can infect neurons and intestinal cells comes from studies of human brain and gut organoids, which are also used to investigate viral infection and replication. The effects of COVID-19 drugs on cardiac activity are being analyzed in 3D human heart models. Commercially produced human lymph node organoids are being utilized to test and develop antibody therapies against SARS-CoV-2. The potential of NATs for combating the coronavirus has been recognized by some regulatory agencies like the United States Food and Drug Administration (FDA) that announced it would use human lung-on-chip models to analyze the safety of COVID-19 vaccines and therapies. In summary, fast, robust, human-relevant NATs have greatly contributed to COVID-19 research and are essential tools for tackling the pandemic.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote is necessary for this report.

24

Twinning towards excellence in alternative methods for toxicity assessment – TWINALT project

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The objections to animal experiments which have accumulated on both ethical and scientific grounds drive the development of new alternative approaches which do not use animals. Despite the plethora of available alternative methods in different fields of safety assessment, there are still either no validated animal-free replacement methods for some toxicity endpoints or they need a thorough improvement/development. Therefore, the international project "Twinning towards excellence in alternative methods for toxicity assessment" (acronym: TWINALT) has been established as a close scientific cooperation of four European partners: Nofer Institute of Occupational Medicine (Poland), Vrije Universiteit Brussel (Belgium), Norwegian Institute for Air Research (Norway) and University of Milan (Italy).

The project aims to significantly strengthen the research position and enhance the scientific and technological capacity of the linked institutions merge and integrate research possibilities with the needs of society at the local, regional, national, European and global level. Five research areas have been identified to be focused on in TWINALT: i) modern methods for cytotoxicity assessment (High Throughput/Content Screening); ii) new cell models in alternative methods (co-cultures, 3D models); iii) standard/modern alternative methods for genotoxicity and carcinogenicity assessment; iv) characterization of cell-nanomaterial interactions under *in vitro* conditions; v) *in silico* methods in safety assessment. Alongside the scientific knowledge exchange, carried out in a form of workshops and on-line and on-site trainings, the project gives the opportunity of developing soft skills useful for young scientists – e.g., scientific writing, project management or using new media to communicate with the society. The project assumes reaching out to the broad auditory, including high-schools, students and commercial stakeholders and opening a dialog with the public, disseminating the knowledge on alternative methods for toxicity assessment.

The project is financed by the Horizon 2020 Coordination and Support action; grant agreement number: 952404.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Mitochondrial toxicity; AOP-driven extrapolation from *in vitro/in silico* data to regulation

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Read-across allows moving from *in vivo*-driven chemical-risk assessment to mechanism-based assessments of data-gaps using new approach methods (NAMs). Thus far, a limited number of these types of *in vitro/in silico*-based mechanistic studies have been presented and used in actual chemical-risk assessments.

We therefore set out 2 case studies to address feasibility and regulatory acceptance [1]. Both case studies are presented using an adverse outcome pathway (AOP)-driven selection of relevant biological processes and associated *in vitro/in silico* NAMs. Each case study is built around a set of biologically and/or structurally similar pesticides targeting mitochondria and assessed the chemicals for their neurological hazard. The present various concepts that can be used to feed into the read-across including analogue versus category approach, consolidated versus putative AOP, positive versus negative prediction (i.e., neurotoxicity versus low potential for neurotoxicity), and structural versus biological similarity.

With these AOP-driven case studies we showed how data from NAMs can contribute to the weight of evidence in read-across approaches, can help to substantiate chemical risk assessment and in the end present mechanistic data in an accessible way to regulatory authorities.

Reference

[1] van der Stel, W., Carta, G., Eakins, J. et al. (2021). *ALTEX 38*, 615-635. doi:10.14573/altex.2103051

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

26

Human-derived *in vitro* test systems of inflammatory bowel diseases as an alternative to laboratory animal models

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Background: Inflammatory bowel diseases (IBD) including Crohn's Disease (CD) and ulcerative colitis (UC) are characterized by a multi-factorial pathophysiology, including genetic perturbations in the regulation of innate immune mechanisms and epithelial barrier integrity. So far, the majority of experimental models implemented in the investigation of the pathophysiological mechanisms of the diseases and discovery of therapeutic substances, include laboratory animal models treated with dextran sulfate sodium (DSS). However, such models lack the organism specific genetic background that occurs in humans. Advances in the field of organoids has allowed *in vitro* culture of human derived intestinal crypts that could be derived from IBD patient biopsies. *In vitro* test systems based on IBD donor lines recapitulate a human-specific background of genetic susceptibility to the pathology. Such platforms could be used for accurate drug screening of targets involved in the development of chronic inflammation based on intestinal epithelial barrier rupture, such as the Aryl hydrocarbon receptor (AhR). Here we present an *in vitro* test system based on generated human IBD enteroids, as an alternative to DSS-treated mouse models.

Methods: Intestinal crypts were isolated from CD and healthy donor biopsies and subcultured to intestinal epithelial organoids according to the previously published protocol by Sato et al. [1]. Intestinal organoids were dissociated to epithelial cell fragments and seeded on a decellularized porcine small intestinal submucosa (SIS) scaffold for the setup of Transwell®-like *in vitro* test systems [2]. Differentiated intestinal epithelial monolayers were tested for barrier integrity using transepithelial electrical resistance (TEER) measurements. Histological analysis with Alcian Blue staining was followed in order to visualize the confluent epithelium and mucus secretion. Gene expression levels of pattern recognition receptors (PRRs), anti-inflammatory cytokines and tight junctions were determined with real-time quantitative PCR. Next to model characterization, the effect of AhR ligands was tested on the gene and protein expression level of specific tight junction markers and regulators of the inflammatory response to demonstrate the relevance of the models in the context of CD.

Results: Histological analysis revealed a confluent epithelial layer in CD and healthy donor-derived Transwell®-like models while mucus secretion was found to be increased in CD donor lines compared to the healthy donors. On the contrary, TEER was found to be decreased in CD-derived epithelial models compared to the control (healthy) ones, accompanied by reduced expression levels of some tight junction genes. Differential expression of pattern recognition receptors was found to occur between the different experimental groups, while donor-to-donor variations were also observed. Treatment with different AhR ligands resulted in a reduction of inflammatory hallmarks and an increase in tight junction gene expression levels on the CD-derived epithelium. Lastly, the protein expression levels of tight junction proteins found to be increased after treatment with the different pharmaceutical compounds resulting in an ameliorated CD phenotype.

Conclusion: Our human intestinal tissue model represents an appropriate *in vitro* test system to further investigate cellular and molecular mechanisms that underline CD pathophysiology. The preservation of the genetic background relevant to the disease and the variation within donors, that recapitulates the complex pathophysiology of the disease, defines our model as a promising alternative to laboratory animal- as well as cell line-based models. Finally, our primary human *in vitro* test systems were used for the selection of drugs targeting AhR, which could be involved in the pathogenesis of the disease. Therefore, these novel models may allow us in future to reduce the number of animal models used for drug testing of potential therapeutics for IBD.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Focus on efficacy: *In vitro* tissue models for preclinical research

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During the development of new therapeutics or medical devices, proof-of-concept is crucial. Currently, animal models are the gold standard for the most part, but animal metabolism can differ significantly from that of humans. This limits the predictive power of animal studies, which explains, among other things, the high error rates of up to 95% during translation from the preclinical to the clinical development phases. In addition, humans are the only natural host for many pathogens, which limits the use of animal models.

To assess the efficacy of novel pharmaceutical ingredients or formulations, we developed standardized *in vitro* tissue models for various barrier organs such as the skin [1], gastrointestinal tract, eye [2], respiratory tract [3] and blood-brain barrier [4]. In addition, various clinical pictures such as a cutaneous wound, various hereditary diseases or infections can be simulated *in vitro*. Furthermore, it was possible to establish meaningful tumor models that mimic not only the tumor cells but also the tumor stroma [5].

The applicability of *in vitro* models for preclinical research will be demonstrated by three examples. (i) The rare skin disease epidermolysis bullosa simplex (EBS) was reproduced *in vitro* using cells carrying the corresponding mutations and showing important aspects of the disease. (ii) Furthermore, we were able to build models of mamma carcinoma and investigate different therapeutic approaches and their effect on various signaling pathways in the cell, in collaboration with the bioinformatics department at the University of Würzburg. The data obtained can be used to predict the effect of various therapies on tumors of different stages. Hence, this can lead to the development of personalized therapy strategies that are specifically adapted to certain patient groups, according to the mutation and their tumor status. (iii) The models were adapted to study pathogenesis of infections for example with *Bordetella pertussis* or *Trypanosoma brucei*.

Taken together, we were able to show that *in vitro* tissue models are suitable for efficacy testing of various pharmaceutical products, thus can complement animal models in preclinical research.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Tracking the validity of animal models for biomedical research

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In the more than six decades since Russell and Burch formulated the 3Rs, surprisingly little progress has been made in the scientific investigation of the validity of animal models for biomedical research. The issue of external validity (i.e., how relevant is the animal model?) is often ignored altogether, with researchers choosing animal models out of habit or on the basis of unchecked assumptions [1]. Worse, the issue of internal validity (i.e., how reliable or reproducible are the findings?) has placed animal-based biomedical research in a negative spotlight with the general public [2].

While most animal researchers may personally be committed to improving their research practices, the micro-motives of well-meaning individuals do not guarantee optimal macro-behavior at the aggregate level [3]. As has been described in behavioral economics, a minority of defectors can disrupt the development of cooperative dynamics. In the present context, defectors would be researchers who persist in substandard research practices, even if this is not out of malice or incompetence. All that is required for the "survival of the un-fittest," or the continuation of substandard research practices, is a research culture with perverse incentives (e.g., short-term gains in publications).

The issues can be vividly illustrated with the use of non-human primates during the COVID-19 pandemic. For instance, "[p]rimate researchers in the United States have banded together for an ambitious monkey study that would do head-to-head comparisons of the leading COVID-19 vaccine candidates" [4], while researchers in China reported that "primary exposure to SARS-CoV-2 protects against reinfection in rhesus macaques" [5], obtaining non-significant results from a complex study with a sample of seven, which proved to be entirely irrelevant, if not plain wrong. The banding together of primate researchers in the United States and the non-significant data from China were both reported in *Science*, arguably the pinnacle of scientific publishing, although anyone with a modicum of scientific reasoning could easily have shown that the primate researchers in the United States engaged in self-serving research bias that ignored the question of external validity, and that the sample of seven rhesus macaques in China did not approach anything like the sample size required for the given research design (as could have been assessed with readily available tools for power analysis, such as G*Power).

The solutions are technically not difficult, but they require a sea change in research culture. In this respect, animal researchers can turn to the human behavioral sciences for a paradigm of a neighboring research field that is making great strides toward the required change in research culture. What is needed is a shift to open science, driven by leading journals and researchers, spreading the guidelines and facilitating the implementation of good practices. These rest on the twin pillars of the pre-registration of experiments and the use of power analysis to compute sample size. These twin pillars can effectively redress the issue of internal validity.

For the external validity, the challenges for animal research are arguably unique. Previous efforts to compare the validity of animal models directly (e.g., [6]) have typically been limited to qualitative, categorical assessment. Moreover, the most important type of replacement – as according to Alexander Pope's verse, "The proper study of mankind is man" – is all too easily forgotten. Here, I would propose that, before engaging in any animal research, researchers should establish the conditions under which it is impossible to work with human volunteers as the preferred animal model. Indeed, even for the most urgent issues or risky experiments, we may still find human volunteers. We just need proper guidelines and broad societal support (e.g., [7]). Then, to the extent that there are still types of research that require the use of animals, it is imperative that we track their validity – this means openly sharing and compiling all the data, and regulating the protocols, not just in academia, but also in industry. Sharing the data, and following academia- and industry-wide protocols, should be the entry point for a license to use animals for research.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Developing new-approach *in vitro* toxicology methods to elucidate the DNA damage mechanisms of nanofibers

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Introduction: Nanofibers, including carbon nanotubes (CNTs) present challenges to human hazard and risk assessment due to their unique geometry and physico-chemical features. Whilst the incentive is to move away from invasive animal testing in favor of more advanced *in vitro* models, there are still significant gaps in the literature relating to CNT mutagenicity and their potential for inducing secondary genotoxic mechanisms, which can only be evaluated *in vivo*. In addition to the lack of data, the current OECD (Organisation for Economic Co-Operation and Development) test guidance (TG) require adaptations to support toxicology testing with engineered nanomaterials (ENMs). This research aimed to establish a harmonized protocol for the *in vitro* mammalian cell gene mutation assay focusing on the hypoxanthine phosphoribosyl transferase (HPRT) gene (TG476) via an interlaboratory trial across two laboratories. The final protocol was then applied to CNTs to determine their mutagenicity. To investigate secondary genotoxic mechanisms the *in vitro* cytokinesis-blocked micronucleus (CBMN) assay (TG487) with lung co-culture models was utilized.

Methods: For the HPRT assay, the test ENMs materials (titanium dioxide, zinc oxide, copper oxide, Nanocyl multi-walled carbon nanotubes and tungsten carbide-cobalt; TiO_2, ZnO, CuO, Nanocyl-MWCNTs, WC/Co respectively) were exposed to human lymphoblast (TK6) cells for 24 h (1-20 µg/mL) and scored for point mutations. For the CBMN assay, monocultured human bronchial epithelial (16HBE14o-) cells and a co-culture of 16HBE14o- with differentiated, human monocytic (dTHP-1) cells were exposed to CNTs (6.25-100 µg/mL) for 24 h. To determine the effects of dispersion upon the biological effect, NM400 and NM401 CNTs were dispersed using either the well-established NANoREG protocol or by Microfluidizer LM-10.

Results: The first interlaboratory trial showed no induction of mutagenicity across both laboratories for ZnO and TiO_2. The second trial (focusing on CuO, Nanocyl-MWCNTs and WC/Co) revealed CuO induced significant, dose-dependent cytotoxicity (36% reduction in viability at 0.5 µg/mL), and a 32-fold increase in mutation frequency. Additionally, Nanocyl-MWCNTs promoted a 6-fold increase in mutagenicity whereas tungsten carbide-cobalt did not induce any significant effects. The interlaboratory data showed good statistical concordance for precision and accuracy. No statistically significant genotoxicity was observed for either CNT type (NM400/NM401) in the HPRT and CBMN assays.

Summary: The successful interlaboratory trial on the HPRT forward mutation assay has delivered a harmonized standard operating protocol, which was then applied for the mutagenicity testing of NM400 and NM401. No significant mutagenicity was observed following 24 h exposure to either CNT type and further, there were no significant differences when comparing the choice of dispersion technique.

The authors would like to acknowledge this research has received funding from the European Union's Horizon 2020 research and innovation program for the RiskGONE (Grant Agreement #814425), NanoSolveIT (Grant Agreement # 814572) and NanoInformaTIX (Grant Agreement #814426) projects.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Human iPSC-derived model to study myelin disruption

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The alarming increase in prevalence of neurodevelopmental disorders exceeding 15% worldwide [1], has become a major public health concern. There is growing evidence that environmental *toxicants contribute to the development of these disorders*. However, the significance of exposure to xenobiotics during developmental stages is not fully understood. The Organization for Economic Co-operation and Development (OECD), defines toxicological test guidelines to evaluate chemicals and assure human health. However developmental neurotoxicity (DNT) is not systematically studied due to the high costs, high number of animals and time-consuming experiments required in the current OECD test guidelines (OECD TG 426 and US EPA 712-C-98-239). On the other hand, there are rising concerns regarding the physiological relevance of extrapolating results from animal studies to humans, indicating that animal studies are suboptimal for many toxicological assessments. The limited testing on DNT, coupled with an increasing need to assess the hazards of thousands of industrial chemicals, has resulted in calls for the development and use of New Approach Methodologies (NAMs) that are based on non-animal approaches. The OECD and European Food Safety Authority (EFSA) are currently developing a Guidance document [2, 3] to evaluate DNT effects based on an *in vitro* testing battery of assays[3] to speed up the replacement of *in vivo* studies in the future.

Myelination of axons in the central nervous system (CNS) is crucial for brain function and is one of the key processes in brain development. The main function of myelin is to protect and insulate axons and enhance the transmission of electrical impulses. *Myelin formation has been considered as one of the most important key events* during brain development, and as a sensitive endpoint for DNT evaluation. However, a myelin assay has not been incorporated yet in the testing battery due to the *difficulty to obtain myelin in vitro*. We have combined the latest advances in cell cultures and induced pluripotent stem cells (iPSC) to develop a human 3D organotypic model for the brain (also called BrainSpheres) [4]. Our model presents unique features making it an ideal tool for myelin studies. Our protocol allows us to generate a high amount of viable BrainSpheres (over 800 per batch), which are not only homogeneous in size and shape but also present reproducible percentages of the diverse cell types. Compounds capable of inducing developmental neurotoxicity by disrupting myelin identified by a systematic review were used to evaluate the relevance of our BrainSphere model for the study of the myelination/demyelination processes. Myelination was assessed by quantifying the co-localization of the immunostaining for myelin basic protein (MBP) and high molecular weight neurofilament protein (NFH), as well as co-localization of proteolipid protein 1 (PLP1) and NFH. Levels of PLP1 were also assessed by Western blot. Results demonstrated that the positive reference compound (cuprizone) and two of the three potential myelin disruptors tested (Bisphenol A, Tris(1,3-dichloro-2-propyl) phosphate, but not methyl mercury) decreased myelination, while ibuprofen (negative control) had no effect [5]. Here, we define a methodology that allows quantification of myelin disruption and provides reference compounds for chemical-induced myelin disruption.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Using an innovative *in vitro* platform to investigate the early effects of oxidative stress and increased pressure on human trabecular meshwork cells

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Primary Open Angle Glaucoma (POAG), which accounts for about 70% of all glaucoma cases, is characterized, anatomically, by an open-iridocorneal angle and, clinically, by a selective loss of retinal ganglion cells (RGCs) and their axons, the cupping of the optic nerve head, and vision loss. In addition, in High-Tension Glaucoma [1], also an increase in the intraocular pressure (IOP) can occur, which is absent in Normal-Tension Glaucoma.

In the conventional pathway of the human eye, the trabecular meshwork (TM) is the first barrier to the aqueous humor, and the loss of its functionality, due to local oxidative stress conditions, causes a vicious circle of damage (e.g., TM stiffening, senescence, apoptosis, and the remodeling of the ECM), which leads to an increase in the outflow resistance and IOP elevation. However, even though IOP is currently considered the only treatable target, increasing evidence suggests that also TM dysfunction *per se* is involved in early RGC death through the pro-inflammatory cytokine secretion [2]. Therefore, identifying the early steps underlying TM damage can provide useful information for the development of TM-targeted therapies to be used in combination with standard anti-hypertensive drugs. Herein, we have reported the effects of oxidative stress and increased pressure on TM human primary cells (HTMC, Cell Application INC.), cultured in our *in vitro* 3D-innovative human-based model (IVOM) [3] which is an *in vitro* platform based on Matrigel-embedded HTMCs and a bioreactor system (IVTech Srl., Massarosa, LU, Italy) equipped with a peristaltic pump (LF, IVTech).

HTMCs were maintained under dynamic conditions for at least 22 h. A day throughout the whole duration of the experiments (i.e., a total of 72 h) [4]. After exposure to 500 µM H_2O_2 for 2h/day, the 3D-HTMCs were subjected to dynamic culture conditions with or without Live Pa, i.e., an auxiliary device to increase the baseline pressure by 10% (from 0.014 KPa to 0.021 KPa) every 12 h. Then, at each checkpoint time (48 and 72 h), the 3D-HTMCs were analyzed in terms of viability, senescence, apoptosis, and mitochondrial function. The 3D-HTMCs, treated with daily doses of H_2O_2 alone or in combination with Live Pa, showed an early significant reduction in cell viability compared to the cells treated with Live Pa alone. Moreover, H_2O_2 conditions induced a marked increase in senescence-positive cells. However, the Live Pa condition alone induced an earlier increase in pro-apoptotic protein levels and propidium iodide (PI)-positive cells compared to H_2O_2-treated HTMCs. Therefore, we can conclude that the combinatory treatment exacerbated the Live Pa-obtained results. Finally, the mitochondrial function analysis revealed that all experimental conditions reduced the HTMC mitochondria activity.

In addition, the potential harmful effects of treated-HTMCs on human 3D-neuron-like cells were evaluated, by culturing retinoic acid-differentiated SH-SY5Y neuroblastoma cells with 50% HTMC-conditioned medium (CM) for a further 48 and 72 h. The analysis of the same endpoints verified on 3D-HTMCs evidenced that oxidative stress-derived molecules released into the HTMC media caused a greater marked impairment of neuron-like cell behavior compared to Live Pa-CM. Moreover, the combination of the two stimuli together enhanced these harmful effects further.

In summary, our IVOM platform proved to be most useful in analyzing the early pathological cross-talk between the damaged TM cells and the neural-like cells, both of which lead to POAG outcomes.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Vitamins' B6 and C enhance the HuIFN-αn3 induction by the NDV ZG1999HDS or Sendai virus

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Interferons (IFNs) are multifunctional glycoprotein/proteins made and released by host cells in response to the presence of different pathogens such as viruses, bacteria, parasites or tumor cells. They allow the communication between cells to trigger the protective defenses of the immune system that eradicate pathogens or tumors. There are two major categories of IFNs, designated as Types I and II. The Type I include IFNs-α, β and ω, while Type II include IFN γ only [1,2]. New Newcastle disease virus (NDV) (strain ZG1999HDS) isolated from lung tissue of the flock of broiler chicken suffering respiratory disease, proved to be lentogenic causing death because of tropism for the lung tissue, is described. It belongs to genotype II of class II NDV [3] The Sendai virus (SV) (Cantell strain) murine Paramyxovirus type I, is a broad used virus to induce the HuIFN-αN3 in the Buffy Coat. The previous experiments were designed to compare the HuIFN-αN3 inducing capability of NDV (strain ZG1999HDS) and SV (Cantell strain) *in vitro* and the enhancing effect of vitamin B6 on it. The NDV (strain ZG1999HDS) possess the interferon inducing capability similar to SV (483 ± 57 pg/mL versus 584 ± 46 pg/mL). HuIFN-αN3 inducing capability of NDV (strain ZG1999HDS) can be enhanced with vitamin B6 to the 3.286 ± 34 pg/mL. In the case of SV (Cantell strain), vitamin B6 enhance its interferon inducing capacity to 4.557 ± 34 pg/mL. Nevertheless, its induced HuIFN-αN3 differs compared to SV (Cantell strain) induced interferon in the absence of the HuIFN-αN3 subtype α14 and in the lower level of the HuIFN-αN3 subtype α1. In the comparative experiments the HuIFN-αN3 enhancing induction of the Sendai virus and NDV with the Vitamin C was found. The effects of ascorbic acid on interferon production and on the antiviral effect of interferon in cultures of human cells were investigated. Ascorbic acid enhanced the interferon levels produced by human embryo skin and human embryo lung fibroblasts, induced by Newcastle disease virus. Leucocyte interferon assayed in lung fibroblasts titrated 0.2-0.3 $\log_{10}$ units higher in the presence of 5 μg ascorbic acid than in the absence of the latter [4]. To test the vitamins B6 and C enhancing effects on HuIFN-αN3 inducing capacity with the NDV ZG1999HDS or Sendai virus, the follwing combinations were used: (1) Priming with 50 IU/mL of HuIFN-αN3 + 20 μL of Vitamin C + 50 HAU (Hemagglutination units) of Sendai virus or NDV ZG1999HDS = 22.973 IU/mL of HuIFN-αN3. (2) Priming with 50 IU/mL of HuIFN-αN3 + 50 μL of Vitamin C + 50 HAU of Sendai virus or NDV ZG1999HDS = 23.700 IU/mL of HuIFN-αN3. (3) Priming with 50 IU/mL of HuIFN-αN3 + 50 μL of Vitamin B6 + 50 HAU of Sendai virus or NDV ZG1999HDS = 37.321 IU of HuIFN-αN3 (4) Priming with 50 IU/mL of HuIFN-αN3 + 100 μL of Vitamin B6 + 50 HAU of Sendai virus or NDV ZG1999HDS = 29.390 IU/mL of HuIFN-αN3 (5) Priming with 50 IU/mL of HuIFN-αN3 + (20 μL of Vitamin C + 50 μL of Vitamin B6) + 50 HAU of Sendai virus or NDV ZG1999HDS = 60.628 IU/mL of HuIFN-αN3; (6)) Priming with 50 IU/mL of HuIFN-αN3 + (50 μL of Vitamin C + 100 μL of Vitamin B6) + 50 HAU of Sendai virus or NDV ZG1999HDS = 73.784 IU/mL of HuIFN-αN3.

It can be concluded that the additive activity of the vitamins' combination of: Vitamin C (20 μL) + Vitamin B6 (50 μL) or Vitamin C (50 μL) + Vitamin B6 (100 μL) on the HuIFN-αN3 induction with NDV ZG199HDS or Sendai virus, can be found. The mechanism of this additive activity is not completely clear.

The research was included into the Project: "Oncolytic Newcastle Disease Virus in the Veterinary Medicine". The authors acknowledge Ivan Čermak from Deltagrupa.Hr, Kaptol 19, 10000 Zagreb, Croatia for the financial support of the experiments.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Knowledge, skills, attitudes and beyond

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The practice of education and training typically refers to three fields when considering animal use and humane, innovative methods (replacement alternatives). The first is education of students and the general public *about alternatives*, primarily those in research and testing. The second is training of scientists, technicians and assessors *in the use of alternatives* for application in research and testing. The third is the formal education and training of students and trainees *through the use of alternatives* at secondary, higher and professional levels. The latter may also include skills training within laboratory animal science, until the transition to animal-free methods is complete. Across these fields, there are different degrees of emphasis between the acquisition of knowledge, of skills, and of attitudes and values; and both commonality and difference between their respective sets of teaching and learning objectives. Legal requirements, developments in technology, a growing sensitivity to the learning process, and the need for ethical commitment and consistency, can inform and encourage new choices of tools and approaches. These can enhance education and training and facilitate broader curricular transformation. This presentation investigates objectives and outcomes in the different fields. It explores both standard objectives and the additional objectives that might be met following a strengthening of vision for what could be achieved in education and training. It also addresses the identification and obviation of the hidden curriculum.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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3D multi-layered epithelial models based on animal-free fibrous scaffolds

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The stromal part is a key element of current tissue equivalents as an alternative for animal experimentation or as advanced biological implants. Due to the lack of synthetic alternatives, most researchers rely on animal derived materials such as collagen [1] as main building block for the respective tissue equivalents. However, besides ethical concerns, biological scaffolds are prone for, batch-to-batch variances, increased costs as well as undesired dependencies. The challenge within the development of suitable 3D scaffold materials is to fulfil and combine requirements such as biomimetic structure, high porosity and bioactivity [2]. Here we present the generation of a highly porous fibrous scaffolds with subsequent cellular biologization. The resulting synthetic-based 3D scaffolds are applicable for a wide variety of current stromal tissue models such as multi-layered epithelia.

A modified electrospinning process generated scaffolds. As a variety of biocompatible polymers are applicable, we focused here on polyamide (12%, HFP) to keep it simple. The porosity was controlled by the addition of NaCl porogen particles in defined proportions and size distributions. The resulting highly porous scaffolds were characterized concerning structure and microscopic mechanical properties by nano indentation. For biologization, the 3D scaffolds were seeded with stromal tissue cells (hMSCs or hFibs isolated from the target tissue). After 2-4 weeks of migration, proliferation, differentiation and matrix synthesis, the scaffolds were stacked to or seeded with epithelial cells (skin, intestine, air way), tumor cells or endothelial cells to form multi-layered and hierarchically structured tissues.

The proportion and size distribution of the porogen particles were defined and the resulting structural properties, like pore sizes, mesh openings and porosities determined. The mechanical properties on the cellular level with a young's modulus of 3 kPa were comparable to conventional hydrogels, e.g., collagen gel. Since the scaffolds allows cellular migration as well as the dynamic rearrangement of the fibers, primary human dermal fibroblasts were able to homogenously populate the scaffold and remodel the structure by secreting ECM components such as collagens. Furthermore, hMSC were able to be differentiated inside the 3D structure (e.g., adipogenic or osteogenic) to transform the synthetic scaffold in further applicable types of biologized tissues. The biologized scaffold material was suitable for combination with human epithelial cells, e.g., primary human keratinocytes and were cultured at the air liquid interface. The resulting multilayered tissue equivalents (e.g., skin, air way, intestine, cornea) based on synthetic fibrous scaffolds were characterized concerning physiological cellular composition and polarization as well the characteristic barrier functionality. Interestingly, the developed setup was able to completely prevent lateral tissue contractions as a major pitfall of common biologic hydrogels, enabling the barrier functionality on the entire surface without edge-related barrier artefacts.

The described modular principle, starting from the synthetic highly porous scaffold (with a variety of applicable polymers), the different biologization possibilities and following combinations among each other or with epithelial/endothelial layers, generated a platform technology which can address a diversity of current tissue models. Thereby a simultaneous replacement of animal derived materials and an increase in tissue complexity can be enabled.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Rational design and evaluation of electrospun scaffolds as cell culture substrates for the cultivation of human induced pluripotent stem cells

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Neurological and neurovascular diseases constitute an enormous economic and social burden and are expected to increase in the upcoming decades due to the aging demography globally. Accordingly, efforts for an improved understanding to treat these diseases are undertaken by basic research and the pharmaceutical industry. However, to treat neurovascular diseases adequately, it is of eminent importance to understand the blood-brain barrier and its specific barrier properties to gain a more profound knowledge of pathophysiological processes affecting the brain and to design new drugs effectively crossing this barrier for the treatment of such diseases.

The blood-brain barrier is formed by a tightly closed cellular interface between the microvasculature and the central nervous system, limiting the transport from the bloodstream to the brain. Its architecture comprises endothelial cells, astrocytes, and pericytes adhering to a basement membrane, a thin fibrillar extracellular matrix layer. This extracellular matrix mainly consists of collagen type IV fibrils and laminin contributing physical support and regulating cellular crosstalk. This highly specialized barrier protects the brain and central nervous system from pathogens and noxae. Therefore, researchers are eager to develop *in vitro* models capable of studying neurovascular diseases and drug transport across this highly selective and effective barrier. Usually, these *in vitro* models rely on the use of immortalized cell lines or primary cells isolated from rodents which are cultivated on stiff and flat Transwell®-membrane inserts as a mono- or co-culture. Despite several advantages including easy cultivation and high experimental reproducibility, cell lines are prone to dedifferentiation associated with the loss of cellular functions such as a low barrier integrity due to the reduction of tight junctions. To overcome these drawbacks, primary cells from rodents can be isolated exhibiting an improved phenotype retention with greater barrier integrity. Nevertheless, primary cells display a greater variability and show inter-species discrepancies limiting their use in human *in vitro* models. Another experimental discrepancy using a Transwell®-membrane insert is the insufficient resemblance of the extracellular matrix, which provides biochemical and biomechanical cues for cell differentiation, proliferation, and migration.

To overcome the disadvantages mentioned above, we have designed and evaluated three-dimensional cell culture substrates which are capable of resembling the extracellular matrix by simultaneously electrospinning two biocompatible and biodegradable polymers. We created fibrillary scaffolds with defect-free fibers featuring diameters similar to native collagen fibrils in the human basement membrane by fine-tuning the electrospinning process. Adjustment of fiber diameters led to a decrease in pore size, which is a major prerequisite for forming a tight endothelial barrier. The rational design approach led to optimal biophysical properties favorable for cell adhesion and ensuring adequate nutrient transport through the electrospun scaffold. Furthermore, mechanical properties of the brain tissue were successfully mimicked after immersing these fibrous matrices in cell culture media, presenting a flexible microenvironment for cell cultivation. These optimized three-dimensional scaffolds provide an excellent structural framework for cultivating human induced pluripotent stem cells to differentiate into brain capillary endothelial cells, which adhered, proliferated, and migrated on the scaffold surface, forming a cellular monolayer. Physiological similarities to the native blood-brain barrier were confirmed by staining the tight-junction protein occludin, exhibiting an *in vivo*-like expression. In addition to the immunostaining, barrier properties were assessed by quantifying the transendothelial electrical resistance revealing a tight barrier formation. These findings were further validated by measuring the paracellular permeability of sodium fluorescein, which mainly depends on the number of expressed tight-junctions, exhibiting low permeability coefficients for the cellular monolayers. By cultivating human induced pluripotent stem cells on a three-dimensional fibrillary cell culture scaffold, we were able to design an *in vitro* model resembling the *in vivo* characteristics of the blood-brain barrier.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethical vote was required.

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Evaluation of the cytotoxic and inflammatory effect of two size-separated fractions of Russian chrysotile asbestos fiber

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Asbestos is currently classified by the IARC as carcinogenic to humans and thus represents a serious occupational and environmental hazard, although in the past and still nowadays it was, and it is, largely employed for several industrial applications due to its outstanding technological properties. In fact, the exposure to mineral fibers induces chronic lung inflammation, with the subsequent development of several lung malignancies. This happens mainly by inhalation, leading to the localization of the mineral fibers in the lung tissue, where macrophages have a fundamental role in both triggering and maintaining the inflammatory response. Subsequently, macrophages release pro-inflammatory mediators in the surrounding microenvironment affecting both alveolar and endothelial cells. For this purpose, a very useful experimental model is the human monocytic cell line THP-1. These cells, in fact, can be easily differentiated into macrophages and co-cultured with different cell types to mimic tissue-specific interactions and challenged by a large variety of stimuli, such as inhalable mineral fibers [1].

Among the asbestos minerals, chrysotile is the most interesting from a commercial and economic point of view, due to its low manufacturing costs and high natural availability. For this reason, although it is currently classified as a carcinogenic substance, this fiber is still mined and exported from countries like Russia, China and Brazil, to cite a few [2]. Since until now no toxicity data were available on a Russian commercial chrysotile asbestos produced in the Orenburg region, we analyzed the acute effects of such mineral fibres, which were mechanically divided into two different size fractions (> or < 5µm length), in comparison to carcinogenic and non-carcinogenic mineral fiber used as controls, namely crocidolite asbestos and wollastonite.

Thus, our experiments were performed on human THP-1-derived M0 macrophages and on HECV endothelial cells, both separately as well as in a co-culture setup, so that the pro-inflammatory mediators produced by fiber-treated M0 macrophages could affect HECV cells in a more physiologically accurate model. Cytotoxicity, ROS production and apoptosis induction were quantified by the opportune tests, DNA damage was investigated by γ-H2AX foci detection and inflammation was studied by cytokine gene expression profile at different time-points.

Both chrysotile fiber fractions were characterized by a significant acute cytotoxic as well as pro-inflammatory effect, with results that were comparable to the well-known damaging effects of crocidolite. In particular, the > 5-µm fraction showed a notably higher cytotoxic potential, with higher levels of genotoxic damage and of induction of cell death, both by plasma membrane damage and by apoptosis. The > 5-µm chrysotile fraction also showed an increased gene expression of TNF-α, MCP-1 and IL-8 cytokines in M0-THP-1 fiber-stimulated macrophages. Furthermore, HECV in co-culture with fiber-stimulated M0-THP-1 cells showed an increased expression of IL-1β, IL-6, IL-8 and Fibronectin only in presence of the > 5-µm fraction as compared to the < 5-µm fraction and to the controls.

Overall, this *in vitro* model allowed to perform a comparative analysis between two size-separated fractions of chrysotile asbestos fibers deriving from the same mineral source and to assess the degree of acute toxicity and inflammatory potential of the two fractions. The results clearly indicate that the longer-fibre fraction is to be considered more harmful to the human health and probably responsible of the carcinogenic potential of this mineral fiber.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

37

Machine learning based drug-induced genotoxicity prediction model by evaluating physiochemical descriptors: Artificial intelligence (AI) approach

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Genotoxins are chemicals which possess the chemical properties of the ability to cause damage to genetic materials within the cells which terms as "drug-induced genotoxicity" [1]. It is particularly important to alert the genotoxin potency of the drugs. OECD approved *in-vitro* methods such as Ames and micronucleus tests can detect genotoxicity of compounds, however these methods are time-consuming and costly [2,3]. Therefore, we need to develop alternative methods for genotoxicity prediction.

We are motivated to develop an *In-silico* model for drug-induced genotoxicity prediction by integrating *in-vitro*/physio-chemical properties of drugs using a machine learning/AI approach.

Total 442 FDA approved drugs have been selected and their *in-vitro/in-vivo* genotoxicity properties are obtained from literatures. Their physicochemical properties, i.e., XLogP3, hydrogen bond donor & acceptor count, rotatable bonds count, topological polar surface area, and molecular weight are chosen as descriptors and their values are obtained from PubChem databases. From this, 331 drugs are considered as training dataset and 111 are considered as test dataset. In this study, genotoxicity prediction models were built using 8 different machine learning algorithms, namely, MLP (multi-layer perceptron), KNN, Logistic Regression, Naïve bayes, Decision Tree Classifier, Support Vector Machine, Extra Trees classifier and Random Forest classifier. Our models classified the genotoxicity values of drugs in binary format where 0 = non-genotoxic and 1 = genotoxic.

We generated Scatter plot and histogram on pair grid revealed that genotoxic and non-genotoxic compounds settled in distinct clusters. Moreover, the highest accuracy 81.98% was obtained by MLP model, and its specificity was 81% and sensitivity was 82%. The other models also classified with accuracy > 72%, sensitivity > 73% and specificity > 59%. Our machine learning approach paves a path to find genotoxicity property of any unknown drug from its physiochemical properties, which is expensive by the *in-vitro* method. *In silico* assessment of genotoxicity may help biologist and medicinal chemist to find safety alerts for their compounds and minimize the time and cost in drug safety assessment and drug development.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The RAT list: A tool for highlighting areas of animal use ready for replacement

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According to Directive 2010/63/EU on the protection of animals used for scientific purposes, "Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure" [1]. Yet, Cruelty Free Europe has identified that many animals are used each year in the European Union (EU) in regulatory tests that have valid replacements.

This situation inspired Cruelty Free Europe to create the Replace Animal Tests (RAT) list: a list of regulatory animal tests that are still conducted in the EU despite having valid a replacement or being redundant. We identified RAT list candidates by analyzing information on numbers of animals used for scientific purposes (primarily official EU statistical reports [2] and the European Commission's ALURES database [3]) and comparing animal use figures to sector-specific regulatory requirements and guidelines.

We arrived at ten tests for the RAT list relating to: skin irritation, eye irritation, skin sensitization, pyrogenicity, botulinum toxin batch potency, antibody production, Leptospira vaccine batch potency, target/laboratory animal batch safety, abnormal toxicity and shellfish toxin batch safety. The actual or estimated number of animals used in these tests in the EU in 2018 was 3,528, 874, 39,646, 30,453, 492,158, 250,000, 4,286, 5,000, 25,000 and 37,272 respectively, totaling 888,217 animals. We identified the sectors responsible for the tests and the Member States in which they were conducted and elucidated the main reasons for their continuation: lack of global harmonization, lack of enforcement, lack of sector- or product-specific validation and/or unclear regulatory requirements and guidelines.

The RAT list highlights regulatory animal tests still taking place in the EU that are overdue for replacement or are redundant, exposes the scale of the problem and gives insight into where and why these tests are still conducted. Cruelty Free Europe and its associate members will continue to use the RAT list as a tool to track trends, educate the public and lobby the European Commission, Member States and regulatory agencies for an end to these tests.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Building virtual cohorts via the integration of public data

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"*In silico* clinical trials" refers to the development of patient-specific models to form virtual cohorts for testing the safety and/or efficacy of new drugs and of new medical devices. This type of approach can be used in all stages of drug discovery, from screening to pre-clinical trials and greatly reduce drug development time and animal testing. *In silico* preclinical trials could potentially address the translational issues in several ways by evaluation of a proof of concept in the early phases of the drug development process [1].

Current *in silico* methods, namely those based on Artificial Intelligence and Machine Learning, depend on possessing great volumes of data, a problem not always easy to overcome. Transcriptomics have the potential of characterizing individuals' biological and disease state [2]. Resorting to the integration of publicly available data is a viable way getting new insights into disease mechanisms, and to simulate patient populations [3]. Nevertheless, public datasets are subjected to different processing and normalization procedures [4] which often prevents data integration and its usage in Big Data experiments.

Although sequencing data sets (e.g., RNA-Seq) are becoming more abundant, the larger proportion of public transcriptomic data streams from microarray (MA) analysis [5]. With the aim building of cohorts of virtual individuals, in this work we present a method for MA data processing and normalization that allows the analysis across previously processed datasets. We accomplish this goal by, first, predicting and reverting mathematical transformations that may have been applied to MA data; and second, applying a normalization technique that considers the expression of House Keeping genes across the whole set of samples in our global database. Our method is shown to decrease batch effects among samples, thus allowing the extraction of biological conclusions from integrated datasets. As means of assessing this approach in a biological context, we've tested a prostate cancer dataset containing data from 63 different GEO experiments with differential gene expression of 817 samples, and the method was capable of reproducing discoveries reported in other prostate cancer-centered publications. This method corresponds to one of the key initial steps to building virtual patients and conduct *in silico* clinical trials. Furthermore, we think that it facilitates the reuse of existing datasets, allowing researchers to perform analysis across larger cohorts, and reducing the needs for extensive animal experimentation required for hypothesis building and validation.

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Submission declaration: PMF, JMGI and JF are employees of Anaxomics Biotech S.L. JMM is employee and co-founder of Anaxomics Biotech S.L. BO is scientific advisor for Anaxomics Biotech but does not perceive any funding support for these services. PMF and JMGI received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 765912 (proEVLifeCycle) and 859962 (SECRET), respectively.
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Ethics vote is not required.

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Penetration of topical aminophosphonates through the dermal barrier – "Skin-on-a-chip" development with an emphasis on animal number reduction

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Aminophosphonates have been a focus of synthetic organic chemistry and drug discovery for many years. They act as structural analogues of amino acids; therefore, they can influence a wide range of biological processes. The results are promising in terms of potential antibacterial, antitumor, anti-inflammatory, wound healing promoting and neurodegeneration protective effects [1]. As many of these affect the skin function, different dermatological applications might play important roles in the future, so the development of formulations using transdermal drug delivery is necessary, because the topically applied compounds are able to bypass the liver first pass metabolism and reduce the possibility of systemic side effects. For that, experimental information about skin permeability is essential, however, according to our knowledge, the transdermal absorption, and the main penetration route (i.e., whether they follow the transcellular, intercellular or transappendageal pathway) of these molecules has not been examined so far. The aim of our work is to study the absorption kinetics of three newly constructed aminophosphonate, which differ in a single functional group. Our result may contribute to the subsequent development of safe and effective topical formulations. As a proper experimental setup, considering the reduction of the number of animals (moreover to contribute to the potential future replacement), a microfluidic skin-on-a-chip diffusion chamber has been developed.

We aimed to study the permeability characteristics of the given compounds in tissues which are as close as possible to human skin. However, since there was no previously available data on the permeability of such molecules in the scientific literature, an *in vitro* pilot study was designed and performed using isopropyl myristate impregnated 0.45 µm pore size cellulose acetate membrane in Franz diffusion cells, with a diffusion surface of 1.767 cm^2. These results supported our hypothesis that all aminophosphonates can penetrate through the membrane under lipidic conditions in a detectable amount. Furthermore, it was also proven that statistically significant difference could be seen between the penetration profile of compounds with and without the specific functional groups. Based on the pilot experiments, we decided to test the molecules' transdermal absorption on *ex vivo* rat skin. The problem of sacrificing surplus rats was eliminated by animal sharing. The animals from which we excised the abdominal skin were used in other experiments as non-treated controls and kept at least 5 days in animal facility before the skin excision.

The first step of *ex vivo* experiments, Franz diffusion cell was used to provide control results gained on a well-known, validated system. Similarly, to the *in vitro* results, the presence of functional groups was a highly influencing factor.

Meanwhile, novel developments of our skin-on-a-chip microfluidic diffusion chamber (MDC) [2] has been established. Skin-on-a-chip devices offer innovative and state-of-the-art platforms for drug penetration studies through artificial membranes, smaller pieces of *ex vivo* skins, alternative skin models (e.g., 3D bioprinted tissues) or even cell cultures with an implemented transwell insert holder. The generalized design is optimized for manufacturing with rapid prototyping techniques such as 3D printing, CNC milling, polymer casting, and laser cutting [3]. Different generations have been manufactured at our laboratory. In all cases, the polymer-based channel assembly is made from polydimethylsiloxane, building up the inflow channel, the receptor chamber, and the outlet channel. The first clamping device was built from polylactic acid; however, it turned out to be too fragile. Hence, the base material was substituted by polyethylene terephthalate glycol, and later with aluminium, which are much more durable. A new, simplified tissue insertion feature has also been implemented.

Currently, the aminophosphonate permeability studies are running in the aluminium MDC using *ex vivo* skin samples. The diffusion surface is 0.5 cm^2, which means that much less animals are needed for the same replication numbers. As a validation, we are comparing the MDC results to the data from the Franz-diffusion cell study. Considering the translational aspects and the flexibility in the MDC design, we are going to test the compounds on human cell cultures or on artificial reconstructed human tissues, which would also contribute to animal replacement beside our well-established procedures aiming to reduce the number of animals.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Computational modelling for cardiac toxicity

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In recent years, cardiovascular toxicity attracts considerable attention from scientists and clinicians since cardiovascular disease (CVD) is one of the leading causes of mortality worldwide. However, except for drugs, the evaluation of the potential cardiotoxic effects of chemicals is poorly addressed and regulated, although environmental contaminants might have an important contribution to the observed health effects.

In silico approaches comprises a series of methodologies, which can play an important role in the reduction, and replacement of *in vivo* experiments that are much more laborious, time-consuming, and expensive. In particular, quantitative structure-activity relationships (QSARs) seek for a relation between structural characteristics of the chemicals and the toxicity. So far most of the QSAR modelling efforts were focused on the prediction of drug-induced cardiotoxicity due to off-target interactions with the cardiac human-ether-a-go-go-related (hERG) potassium channel and several freely available tools based on the QSAR methodology are nowadays available to scientists to screen *in silico* for this type of effect (e.g. [1,2]).

Here we will present a new model developed using different machine learning algorithms. The application of i) the best practices for data curation; ii) VSURF feature selection method and iii) the Synthetic Minority Oversampling Technique (SMOTE) to properly handle the unbalanced data, allowed developing highly predictive models (BAMAX = 0.91, AUCMAX = 0.95).

Recently Krishna et al. [3] provided an overview of cardiotoxic effects of chemicals for both drugs and environmental chemicals and their associated mechanisms of action, including hERG inhibition among others. Based on these data the aim of this contribution is to better explore the relevance of the already existing models to: 1) Characterize the chemical space covered by the hERG models in terms of prediction ability for drug and non-drug compounds (e.g., pesticides, PAHs, etc.); 2) Profiling compounds according to most represented mechanisms of action for cardiotoxicity (such as oxidative stress, mitochondria dysfunctions and tyrosine kinase inhibition) to preliminary address these other toxicity mechanisms *in silico*.

This analysis will set the basis to improve our current modelling capabilities of cardiotoxicity and can provide useful new models for an AOP-informed screening of chemicals for cardiotoxicity.

This work was supported by the European Union's Horizon 2020 research and innovation program (grant #101037090). The content of this manuscript reflects only the author's view, and the Commission is not responsible for any use that may be made of the information it contains

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Increasing confidence in NAMs through the integration of *in silico* and *in vitro* data: Pyrrolizidine alkaloid (PA) case study

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This presentation will introduce the *in silico toxicology protocols* describing the formation of an international consortium including regulators, government agencies, industry, academia, model developers, and consultants across many different sectors to develop harmonized approaches to apply *in silico* predictions and their integration with experimental data. The consortium developed a protocol framework that outlines how to select, assess, and integrate *in silico* predictions alongside experimental data for defined toxicological effects or mechanisms. This includes a new methodology for establishing the confidence of the final assessment [1]. These protocols are being created for a number of major toxicological endpoints and this presentation will outline the protocol framework and the concepts using the *genetic toxicity in silico protocol* [2] applied to pyrrolizidine alkaloids (PA) as a case study. PAs are a large group of natural toxins synthesized by different plant species that can contaminate food crops, several of them known to be carcinogenic and mutagenic. Therefore, increasing the confidence of their mutagenicity assessment would bring significant benefits in supporting their grouping and is of high importance for the risk assessment.

It will be described how the principles outlined in the protocols can be practically used to perform hazard identification, how computational models and *in vitro* data covering genotoxic mechanisms for PA can be integrated, as well as the role of expert review throughout the complete process. Finally, the confidence associated with the final assessment after analyzing the completeness, reliability, and relevance of all the information will be discussed and the benefits associated with the use of *in silico* protocol principles and the lessons learned from this process will be illustrated.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Effect of group size and cage cleaning interval on male aggression level and wellbeing in two mouse strains

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Aggression between males can be a serious welfare problem for maintaining mice in the laboratory, as aggression can lead to stress, pain, and even fatal injuries [1]. Several factors have been identified as contributing to aggression in group-housed male mice, including strain [2], and cage cleaning regime [3]. However, still little is known about optimal group sizes and the impact of cage cleaning intervals on male aggression and wellbeing. Increasing our understanding of aggression in male mice and how housing conditions can reduce aggression could have a large welfare impact on a significant number of laboratory animals.

Here we experimentally manipulated male group size (single-, pair-, or triplet-housing) and cage cleaning interval (weekly versus bi-weekly) in C57BL6/N inbred and B6D2F1 hybrid mice and assessed male behavior and welfare in both strains. We used 84 virgin males from each strain and randomly divided them into a single- (N = 14 cages), pair- (N = 14 cages), or triplet-housing group (N = 14 cages). Half of the cages within each group were then assigned to a weekly cleaning interval and the other half to a bi-weekly interval. All cages (type IIL, 36.5×20.7×14 cm) contained wooden bedding, a cardboard tube, and cellulose pads as nesting material. Males were 8 weeks old at the beginning of the experiment, which lasted for 8 weeks. We performed behavioral observations (evening and post cage cleaning observations) to determine the level of aggression, the incidence of barbering (fur and whisker trimming) and co-sleeping rates during this time. Furthermore, we repeatedly assessed fecal corticosterone metabolite (FCM) levels over the course of the experiment to estimate animal stress levels.

To determine the direct impact of cage aggression on individual animals, we calculated active and passive attack rates per male (i.e., mean number of initiated and received attacks per week) in pair- and triplet-housed males over the course of the experiment. Passive attack rates varied significantly over time, and B6D2F1 hybrid males faced on average more attacks from their cage mates than C57BL/6N inbred males. Even though attacks occurred more frequently on days with cage cleaning compared to days without cage cleaning, the mean number of received attacks per male did not differ between the weekly versus bi-weekly cleaning interval. In addition, we found no difference in passive attack rates between pair- or triplet-housed individuals. As expected, FCM levels were positively correlated with active and passive attack rates of individuals.

Overall, 20% of the experimental mice showed barbering behavior. We found that group size significantly affected barbering prevalence, as single-housed mice showed almost no barbering (4%), pair-housed mice had a barbering prevalence of 11% and triplet-housed mice of 35%. Cage cleaning interval did not affect barbering behavior, and neither did strain. No difference in the level of observed aggression was detected between barbered and non-barbered mice as active and passive attack rates were comparable between groups. Furthermore, barbered and non-barbered mice did not differ in their mean FCM levels.

The frequency of co-sleeping differed significantly between strains and C57BL/6N inbred males showed more co-sleeping than B6D2F1 hybrid males. Interestingly, co-sleeping was affected by cage cleaning interval, and was higher in groups with a weekly compared to a bi-weekly interval. No difference in co-sleeping behavior was found between pair- or triplet-housed males, but we found that high rates of co-sleeping were associated with low attack rates in males.

Finally, we found that mean FCM levels were lower in single- compared to pair- or triplet-housed mice, whereas pair- and triplet-housed mice did not differ from each other. Cage cleaning interval had no effect on FCM levels and there was no difference in FCM levels between C57BL/6N and B6D2F1 hybrid males.

In summary, we found that B6D2F1 hybrid males are more aggressive than C57BL/6N inbred males. A more frequent cage cleaning interval did not cause higher aggression, or stress levels over the course of the experiment, even though cage cleaning lead to a short peak in agonistic behavior. Our short-term social isolation seemed to have no additional impact on wellbeing, as single-housed males showed lower FCM levels, and fewer barbering behavior compared to co-housed males. The incidence of severe fighting with visible injuries and wounds was low in our study occurring in only 3% of co-housed mice. Pair-housed and triplet-housed mice did not differ in their FCM levels, or in their intra-group aggression level, suggesting that both group sizes equally allow the formation of stable social hierarchies. Given that co-sleeping was associated with lower stress hormone and aggression levels in our study, co-sleeping might be used as an indicator of animal welfare in group-housed male mice.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote on this study.

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Evaluation of Charité 3R measures to implement 3Rs in biomedical research at Charité using online surveys and personal interviews

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Charité – Universitätsmedizin Berlin is one of the largest university hospitals in Europe, with a strong educational and research activity in addition to the central mission of patient care and treatment. Charité's biomedical broad research focus involves conducting of animal experiments using nearly 52,000 animals per year (2021). In 2018, Charité 3R was launched as an institution of the Charité faculty to implement the 3Rs in biomedical research in an advanced and comprehensive manner. Charité 3R aims to improve both animal welfare in animal experiments and the development of alternative methods to replace animal experiments, thus fostering successful implementation of the 3Rs in biomedical research. Charité 3R is equipped with a budget to fund research projects and infrastructures that directly implement replacement, reduction, and refinement measures. Like other 3R centers and initiatives in Europe, Charité 3R is challenged by the difficulty to measure the success and the impact of its activities.

Since evaluation on measures is crucial to adjust future activities, we conducted three approaches for direct feedback on Charité 3R perception. (i) During summer 2021, Charité 3R surveyed Charité employees to determine the effects and efficacy of general Charité 3R measures. This survey was conducted anonymously with scientific staff members, animal welfare officers, animal caretakers and technical staff members. To trace the impact of the Charité 3R intramural research funding, (ii) an online survey among applicants and recipients of Charité 3R research funding and (iii) a set of personal interviews among Charité 3R funding recipients were performed.

The general survey of Charité 3R perception addressed all fields of action: communication, education, and support as well as overarching questions regarding the funding activities. All three Rs were rated with similar relevance, supporting Charité 3Rs approach to address all the three Rs. 41% of survey participants mentioned educational measures as service they have used so far and current Charité 3R measures were predominantly evaluated positively although not all measures reached full visibility within the Charité research community. Interestingly, when the impact of measures was questioned using Likert scale format, a high degree of uncertainty came up, indicating that main future tasks will focus on expanding transparent reporting and continuing communication on the outcomes of Charité 3Rs measures. In addition, clear topics for future action were suggested by the survey participants, too.

From 2018 to 2021, Charité 3R funded refinement and replacement projects, supported 3R technology platforms, offered additional funding for the implementation of 3R in already existing third-party funded projects and substantially supported the initiation of 3R networks at Charité. To approximate the effect of these Charité 3R funding lines, measurable outputs were surveyed among 107 Charité 3R funding recipients. Charité 3R research grants supported 39 publications in peer-reviewed journals. 94% of the funded projects resulted in or planned for national or European wide third-party follow-up funding. Collaboration substantially increased in 75% of the funded projects. In order to get a deeper insight into the implementation of the 3R aspect following Charité 3R funding, six interviews with principal investigators of funded project were performed and analyzed. In addition to measurable output like publication and follow-up grant application, the PIs were asked for (i) their motivation to conduct 3R projects, (ii) for the impact of the funding to develop and implement an alternative method in future research, (iii) their measures to disseminate the results from the funded project, (iv) impact of the funding for the generation of a 3R awareness. The higher scientific value (higher translatability or reproducibility) of a model was identified as the strongest motivation to establish alternative methods for most replacement projects. Both survey and interviews addressing Charité 3R funding were a valuable tool to identify the sustainable impact of the funding. For future setup of funding, we will continue to interview grant recipients to sharpen the objectives of the funding lines and to interrogate the need of the researchers.

In summary, online surveys and personal interviews delivered valuable feedback from stakeholders and gave an insight into the perception of the 3Rs topic and Charité 3R within the Charité research community.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest.
Statement on ethics vote: An ethics vote was not required.

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3R-SMART: Schooling platform for methodological approaches to reduce animal tests

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The Directive 2010/63/EU firmly strengthens the adoption of the 3R principle for the use of animals for scientific and educational purposes. In order to support 3R research activities from different stakeholder in the field, the University of Veterinary Medicine Hannover and the Philipps University of Marburg are collaborating in a BMBF-funded project called 3R-Smart a 3R Schooling platform for Methodical Approaches to Reduce animal Tests.

The goal of the project is to support 3R-research activities by establishing an open access platform that aims to provide training tools to replace, reduce, or refine animal experiments. The platform is tailored to the needs of students, PhDs, graduated scientists and technical staff of universities, public institutions, regulatory authorities, companies, and ethics committees.

The 3R-SMART website (www.3R-smart.de) has been online since March 2020.

The website not only offers instructive content about alternative methods in form of texts, explanatory videos or recordings of lectures, but also news and updates, a calendar for upcoming events and a forum that offers the opportunity to exchange ideas in the field of 3R.

3R-SMART is an open platform that allows different 3R stakeholders to present their 3R efforts within the platform. In addition, 3R SMART will provide open educational resources and plans to offer 3R Seminars or other training resources (fee based) for continuing professional development (CPD). Thus, 3R projects will be presented within the public platform of 3R SMART, addressing a broad audience while also offering teaching resources and fee-based CPD. In line with the idea to disseminate and advance knowledge about the 3Rs we are working on developing a curriculum that can for example be integrated in laboratory animal science courses to help prove 3R-competencies.

Currently we are working on interactive maps to introduce the 3R centers in Germany and Europe in order to present and set up a Europe-wide 3R network that supports the application of 3R methods.

In this way 3R-SMART can provide significant and sustainable support for a wider implementation of 3R-methods. This will hopefully lead to a reduction in the number of animals used for scientific and educational purposes.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Preregistration of animal research protocols: Insights into preclinicaltrials.eu 3-years development

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Poor translational rates raise concerns about the quality and relevance of animal studies, supporting the 3Rs movements to improve animal welfare and reduce or replace animals when possible. Among different initiatives, preregistration, i.e., registering a research protocol before the start of the experiment, recently arose as a potential intervention to improve study quality and facilitate reduction. Since 2000, preregistration has been recommended for clinical trials, where it has proved beneficial but remains sporadically used for animal research.

In 2018, we launched the first online registry dedicated to the preregistration of animal study protocols: PreclinicalTrials.eu (www.preclinicaltrials.eu). The platform aspires to provide an open and comprehensive overview of all animal studies, including those with lower chances of being published (e.g., interrupted studies, neutral data). By giving open access to these data, this initiative supports the reduction of publication bias, aims to prevent involuntary duplication of animal studies, and may help the community to utilize valuable data that is often overlooked or dismissed. Furthermore, recording key methodological features *a priori* makes comparisons between the planned approaches and the completed studies feasible. This process helps to reduce biases and malpractices (e.g., selective outcomes reporting, HARKing) and might even create awareness regarding robust internal validity (e.g., randomization, blinding) and reporting standards.

Since the platform's launch, the PreclinicalTrials.eu team focused its effort on increasing awareness and facilitating the preregistration process, for which it received international recognition. Within these three years, the discussion on preregistration intensified substantially in particular with the rise of comparable registries and the increasing support for preregistration in the Netherlands. Notably, this led to the funding of the platform by the Dutch government. Following this event, several funding bodies set up pilots of mandatory preregistration, and the group received further support from animal welfare bodies and scientific institutes.

Despite this growing effort, the number of preregistered studies is relatively low, revealing room for improvement [1]. As of May 2022, the platform holds 115 published protocols from 25 different countries. As a next step, the Preclinicaltrials.eu team focuses on understanding the effect of preregistration and the underlying factors influencing its application; in an attempt to improve uptake rates. The team also seeks to create incentives and encourage quality gatekeepers, such as journals and funders, to partake in its implementation. With the help of international collaborators (e.g., Center for Open Science, Animal Study Registry) and its ambassadors' network, the PreclinicalTrials.eu team is committed to spread preregistration awareness further and participate in making it the new standard.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

47

In vitro assay-based drug efficacy testing for the treatment of inflammatory bowel disease (IBD)

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Crohn's disease and ulcerative colitis, the two main types of inflammatory bowel disease (IBD) diminish the life quality of an increasing number of patients [1]. The understanding of the disease, the testing of novel drug substances for treatment of acute inflammatory phases and prolonging the remission phases have led to numerous animal experiments with IBD-related models. In order to decrease these animal experiments, reliable *in vitro* assays with the ability to predict human data are highly required.

In our study, we set up an *in vitro* assay to simulate the inflammatory state of IBD for drug screening. The predictivity for human data was evaluated by application of four known drugs related to the severity level of the disease depictured by the "treatment pyramid" for IBD. The assay consists of the epithelial cell line Caco-2 and monocyte-derived macrophages (MDM), that are arranged in a co-culture system, which can be successfully inflamed by stimulation with lipopolysaccharides (LPS). We evaluated the *in vitro* readouts: TEER, cytokine release (TNF-α, IL-6, IL-8) and apparent permeability coefficient (P_{APP}) by calculation the drug efficacy. The drug efficacy was defined as range between LPS control (fully inflamed, therefore 0% drug efficacy) and medium control (not inflamed, therefore 100% drug efficacy).

The release of TNF-α, IL-6 and IL-8 was identified as fast readout for efficacy testing ("yes-no response"). The readout TEER showed the best *in vitro in vivo* correlation (IVIVC) to the severity level represented by the 'treatment pyramid of $R^2 = 0.68$. Based on the concept of an Adverse Outcome Pathway (AOP) framework, which is suggested for the establishment of new *in vitro* models in the OECD guideline: "Guidance Document for Describing Non-Guideline in Vitro Test Methods" [2], we described the *in vivo* effect of drug treatment as "Efficacy Outcome Pathway (EOP)" to evaluate the assay relevance. The EOP includes like the AOP the molecular interactions, the cellular, organ, organism and population responses. This *in vitro* assay offers the possibility for drug screening that might be further validated.

The project was financially supported by the European Regional Development Fond.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Dry powder aerosols crossing the pulmonary barrier *in vitro* – Powder atomizer aims for predicting human pharmacokinetic profiles

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Bioavailability of inhaled dry powders is influenced by many different factors such as aerosol characteristics, chemical properties, and the interplay with different cellular and non-cellular barriers. Translating this process to an *in vitro* assay is a challenge which requires the combination of a mechanical instrument [1] with selected cellular components [2] to predict human data.

The Powder Atomizer is a new developed instrument concept which allows for dry powder aerosol deposition, biological barrier integration and continuous sampling from the basolateral compartment. First experiments were performed with Calu-3 as cellular barrier, human mucus [3] as a non-cellular barrier and size-separated sodium fluorescein and salbutamol sulfate. Transport studies were evaluated with focus on the fraction absorbed *in vitro*. Barrier integrity of Calu-3 was investigated by measuring Transepithelial Electrical Resistance (TEER). Significant differences were found depending on the applied bio barrier. Observed transport curves were similar to pharmacokinetic time profiles. This might be explained by the increased transport after drug deposition followed by an *in vitro* clearance due to the buffer flow in the basolateral compartment.

We will investigate further compounds and compare their *in vitro* transport profiles with known human data. Our aim is to identify a setup which allows for a prediction of human data by our *in vitro* approach.

The project was financially supported by the Central Innovation Program for small and medium-sized enterprises (SMEs), project "ALPDA" Germany. Marius Hittinger and Janina Osti were financially supported by the INNO-KOM project "Kombipulmonal" (FKZ 49MF200045).

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

49

Sharepoint refinement: Internal platform for improved refinement implementation

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Charité – Universitätsmedizin Berlin is one of the largest university hospitals in Europe with strong educational and research activities in addition to the central mission of patient care and treatment. The broad research spectrum in biomedicine with various scientifically active institutes and departments requires about 50,000 animals in experiments per year. Making this *in vivo* research as animal welfare adapted and gentle as possible is one of the goals anchored in the 3Rs principles [1] – Refinement.

In 2018, Charité 3R was launched as an institution of the Charité faculty to implement the 3Rs in an advanced and comprehensive manner, to develop non-animal methods and to improve animal welfare in animal experiments.

About 1,500 researchers, technicians, animal caretakers and veterinarians are involved in experimental procedures, husbandry, genetically engineering and breeding. These people are distributed over five campuses, with high staff fluctuation within all research groups and facilities. Therefore, preservation and improvements of methods as well as a continuous transfer of knowledge is of great importance, but also challenging.

In addition, new insights from the field of laboratory animal sciences and 3Rs [2] as well as rapid changes in legal and administrative requirements pose major challenges for scientists when it comes to planning, applying and conducting animal experiments [3].

A group of Charité animal welfare officers, Charité scientists and the Charité 3R office joined forces to tackle these challenges: An internal data sharing online platform called "Sharepoint Refinement" was built up to provide a fast acting, flexible structure for the implementation of refinement of animal experiments. This platform gives easily accessible support by providing practical instructions like videos, protocols, local standard procedures and contact persons for further support in refinement techniques. It also aims at connecting Charité animal experimenters to share their methodological expertise as well as equipment in an uncomplicated manner to master the challenging situation and the high goals in animal welfare. Since it is an in-house solution, people involved in animal experiments find a protected, non-competitive area to share their knowledge and questions. It is also meant to increase the awareness for selected external refinement resources as well as for other relevant Charité facilities, e.g., for animal imaging.

The Sharepoint Refinement is a living structure – it is constantly expanded, the content is adjusted to the people's needs and it will serve as a base for further exchange and other activities, thus helping to implement refinement and other 3Rs aspects in future animal experiments. Its added value is that presented protocols on, e.g., a single handling method are completed by contact information for help and experience reports from colleagues next door: for example, a short video depicting how animal and experimenter learned a procedure, or a protocol adjusted to a specific experimental set-up, as well as honest report about pitfalls.

Overall, this protected internal Charité online platform with a low threshold access will strongly support implementation of contemporary refinement methods in biomedical research at Charité.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

50

Young TPI: The Dutch initiative for empowering the new scientific generation to go animal testing-free

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The initiative *Transitie Proefdiervrije Innovatie* (TPI) comprised by the Dutch government, companies, and academia deliberately chose in 2018 to shift the focus from reducing animal studies to increasing animal-free innovations. However, transitions usually require one or two generations [1]. The transition to animal-free innovations will be no different. Therefore, we can expect young professionals and students of today to facilitate and accelerate this shift. As a result, it is critical to involve them in the transition early in their careers and to make them aware of what is possible in the field of animal-free methods.

Young TPI is born as an enabler for providing young professionals and students with the space to have an impact. Young TPI is a network aiming to empower the new scientific generation to use their thinking and power in relevant ongoing animal-testing-free initiatives and existing forms of consultation. The network focuses on the breadth of research, from fundamental research to translational, applied, and regulatory research, to find out how we may advance the acceleration towards animal-free methods. It also focuses on understanding the factors behind the acceptance and implementation of animal-free methods. We want to accomplish this through three pillars: stimulate the transition (e.g., working with collaborators, ambassadors, and creating incentives for a change), raise awareness & show the possibilities (with lectures and courses) and create a network & share experience.

Starting in February 2022, the network already attracted almost 40 young scientists and students, and keeps gaining members weekly. Currently, the network focuses on the Netherlands, with future activities such as physical lectures, think tanks, working groups, or lab visits. However, Young TPI intends to exert its influence in Europe in the upcoming years to empower all young scientists to go animal-testing-free.

Reference

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

51

Advancing 3Rs: The National Research Programme 79 of the Swiss National Science Foundation

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In 2021 the Swiss National Science Foundation (SNF) has launched the National Research Programme 79 "Advancing 3Rs – Animals, Research and Society" (NRP 79). With a total budget of 20mio CHF, it is the aim to innovate and implement 3R strategies and reflect 3R research within a time frame of five years. In this talk, the main objectives and initiatives planned in the NRP 79 will be presented. The NRP's overarching goal is to develop innovative methods and instruments that will ensure that the number of animal experiments as well as the number of animals used in university and private-sector research in Switzerland will be measurably and significantly reduced. Three main research modules were identified, namely "innovation," "implementation" and "ethics and society." Whereas "innovation" and "implementation" focus on improvements in practice, the third module "ethics and society" brings the perspective of the humanities and social sciences into the NRP 79. It aims to address the ethical, psychological, legal, social, historical, cultural, and economic aspects of animal experimentation and 3R research. With these three focal points (innovation, implementation and ethics and society) the NRP 79 promises, on the one hand, to provide new insights and progress in 3Rs research and, on the other hand, to design forward-looking strategies for 3Rs research in general. This is done against the background of recent societal developments in the human-animal relationship and its political regulation.

Submission declaration:
This abstract has also been submitted and accepted at the FELASA Conference 2022.
Statement on ethics vote: Not applicable.

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Lysosomal trapping measurement in multiple cell types using high content screening system

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To obtain relevant *in vitro* pharmacokinetic data for *in vivo* predictions, it is essential to determine correct intracellular free drug concentrations. Lysosomal trapping has been described to affect intracellular free concentration of basic lipophilic drugs that results in modified bioavailability, clearance and DDI of these compounds [1]. The potential to minimize animal testing and unnecessary human studies, and time and cost effectiveness, are great advantages of *in vitro* tests compared to *in vivo* trials, and their improvement is important for the replacement of *in vivo* models. To evaluate lysosomal trapping of drugs different methods are available including the detection of lysosomal dye accumulation by fluorescent detection as well as, determination of intracellular concentration of a drug in the presence of lysosome inhibitors [2]. Our goal was to set up an assay for the prediction of lysosomal trapping in HEK293, Caco-2 and human hepatocyte cells by using High Content Screening System and Microplate Reader. We used Lysotracker Red dye for the indirect detection of lysosomal trapping, where the lysosomotropic compound extrude the dye from the lysosomes. The resulted reduction in signal intensities is the basis of IC50 calculation. We chose chloroquine, propranolol and imipramine as reported lysosomotropic compounds and rosuvastatin as negative control [1,2]. Ammonium-chloride was used as a control for total inhibition of lysosomal function [2]. The IC50 values of chloroquine, propranolol and imipramine were similar in HEK293, Caco-2 and human hepatocyte cells and were also comparable to the available literature data based on immortalized cell line and rat hepatocytes [1,2]. For our knowledge, here we report lysosomal trapping IC50 values for these pharmaceuticals in Caco-2 and primary human hepatocyte cells for the first time. The used concentrations of these compounds (100-0.13 µM) showed no cytotoxic effect with LDH or Resazurin based method. While our lysosomal trapping detection was successful with the High Content System, the measurements with the Microplate Reader proved to be less sensitive and reliable. In conclusion, the developed assay using High Content Screening System is a superior method to evaluate lysosomal trapping with multiple cell types and compounds.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Differentiation of specialized epithelial cells in porcine intestinal organoids

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Due to physiological and anatomical similarities to the human gastrointestinal tract, the pig is a popular model animal for human intestinal diseases. Despite these similarities, there are also differences in pathophysiological reactions, such as to an infection with *Yersinia enterocolitica*. While infection with this bacterium is usually asymptomatic in pigs, it can cause febrile gastroenteritis in humans. The use of intestinal organoids is a possible way to investigate the still unexplained pathomechanisms behind this. Under normal circumstances, depending on the part of the intestine, about 80% of intestinal epithelial cells are absorptive enterocytes, about 5% are goblet cells, and less numerous are enteroendocrine cells, Paneth cells, and crypt-based columnar stem cells. In this study, organoids are differentiated such that the physiologically rarer specialized epithelial cells are more abundantly expressed. These organoids are to be used in follow-up *in vitro* studies to investigate the interaction between pathogens and these specialized epithelial cells.

Differentiation experiments were performed on crypt-derived porcine intestinal organoids as well as on human intestinal organoids differentiated from induced pluripotent stem cells. The organoids were cultured in four differentiation media for 72 hours each in comparison to a stem cell niche promoting long-term culture medium. To achieve a more diverse cell population IGF-1 and bFGF were added to the medium [1]. In another experiment Notch inhibitor DAPT was added to induce enhanced expression of goblet cells [2]. In a third experiment DAPT, Wnt inhibitor IWP-2 and MEK inhibitor PD0325901 were added to increase the expression of enteroendocrine cells with or without epidermal growth factor (EGF) [2] and in a last experiment receptor activator of NF-κB ligand (RANKL) was supplemented to induce differentiation to Microfold (M) cells [3]. Each experiment was repeated three times.

The organoids were analyzed via RT-qPCR for expression of cell-specific markers such as LGR5 (intestinal stem cells), Lysozyme (Paneth cells), MUC2 (goblet cells), Chromogranin A (enteroendocrine cells), SpiB and GP2 (human M cells). Furthermore, immunofluorescence staining is performed using markers for Villin (brush border), PCNA (proliferating cells), SOX9 (stem cells and progenitor cells), Chromogranin A (enteroendocrine cells) and Cytokeratin 18 (porcine M cells).

Upregulation of cell-specific marker genes in porcine and human organoids exposed to differentiation factors could be verified via RT-qPCR. Immunofluorescence staining is still ongoing.

First results show species-specific reaction to treatment with RANKL: porcine organoids showed increased crypt proliferation in contrast to human organoids, indicating stronger expression of intestinal stem cells. These morphological findings could be verified via RT-qPCR, which showed increased expression of intestinal stem cell marker LGR5 in porcine organoids after treatment with RANKL.

This project is funded by the H. Wilhelm Schaumann Stiftung.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote required.

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Evaluating the toxicity of sea-dumped conventional and chemical munitions to fish and human cells using a combination of cell viability assays

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Following World War I and II, colossal amounts of munition, including conventional explosives and chemical warfare agents, were sea-dumped in coastal and off-shore sites all over the globe [1]. As a consequence of decades of exposure to seawater, munition shells are expected to be highly corroded thus allowing the release of toxic chemicals to the environment [2]. While the growing interest on the ocean as a new economic frontier and the increasing investments in the blue economy highlight the need for remediation of such dumpsites, the toxic potential of the dumped munition and related chemicals to human health and marine organisms is still largely unknown. However, valuable information to address this data gap can be obtained via the incorporation of new approach methodologies (NAMs). Here, both *in vitro* and *in silico* methodologies were applied to shed light on the impact of these legacy pollutants.

In this study, first insights on the cytotoxic potential of conventional and chemical munition (and related chemicals frequently detected in environmental samples) to rainbow trout gill (RTgill-W1) cells and human colon adenocarcinoma (Caco2) and hepatocellular carcinoma (HepG2) cells is provided.

Chemicals were selected based on their frequent detection in environmental samples and the fact that their toxicity to fish and humans is unknown. Specifically, three chemical warfare agent related chemicals, i.e., thiodiglycol, 1,4-oxathiane and 1,4-dithiane, all degradation products of sulphur mustard, were tested at six different concentrations ranging from 3.13 to 100 mg/L while four explosives related chemicals, i.e., 2,4,6-trinitrotoluene (TNT), 1,3-dinitrobenzene (1,3-DNB), tetryl and picric acid, were evaluated at concentrations ranging from 1.56 to 50 mg/L.

The cytotoxicity of the selected chemicals to RTgill-W1 was assessed following the guidelines described in OECD test guideline 249, following 24 hours exposure [3]. Three cell viability assays were combined to assess the effect of the chemicals on the integrity of the lysosomal membrane, the metabolic activity and the integrity of the cell membrane, thus providing information on the mode of action of each chemical. The same array of assays was used to assess the cytotoxicity of these chemicals to Caco2 and HepG2 (with minor adaptations to the seeded cell density and exposure period) using cell viability measured after 24 and 48 hours of exposure as endpoint.

The obtained results allowed the estimation of the no-observed effect concentration (NOEC), lowest-observed effect concentration (LOEC) as well as the effect concentration (EC) resulting in 10%, 20% and 50% loss of cell viability (when compared to the controls). In general, the results show that the tested explosives and related chemicals are more toxic to both fish and human cells than that of the chemical warfare agent related chemicals. Further, specific effects to one of the performed assays, potentially indicating chemical mode of action, was evaluated and incorporated in known adverse outcome pathways (AOPs) in order to identify the cascade of events leading to an adverse outcome. Overall, the combination of *in vitro* and *in silico* methodologies here applied allow an efficient, animal free and more environmentally sustainable assessment approach.

Ultimately, the gathered data provides a first insight into the cytotoxic potential of seven relevant sea-dumped munition related chemicals. These insight and data can be used for risk assessment purposes.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Implementing the Directive with a focus on severe suffering: Good practice for openness and refinement

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It is widely accepted that openness and transparency around animal use in research and testing should include clear information about harms to animals, and what has been done to reduce pain, suffering and distress. This helps citizens to gain a full understanding of the research that is done with public money, and in the public's name. It is also important with respect to sharing good practice for Refinement, enabling scientists to learn from one another and further reduce suffering.

Any level of suffering is a concern, but "severe" suffering is of greatest concern to the public, scientific community and regulators. Transparency around procedures with the potential to cause severe suffering is essential, including the decision-making processes that determine justification and necessity, and efforts that are made to reduce, avoid and refine severe procedures.

Directive 2010/63/EU requires Member States to ensure that any possible pain, suffering, distress or lasting harm is reduced to the minimum (Article 4.3) [1]. With respect to severe procedures, Directive Article 39 requires these to undergo a retrospective assessment. This includes an evaluation of the harms inflicted on animals, and any elements that may contribute to the further implementation of replacement, reduction and refinement. Member States should ensure that the non-technical project summary is updated with the results of the retrospective assessment (Article 43.2).

Complying with the above will require effective systems for analyzing and refining severe procedures at the project planning stage, ensuring effective welfare assessment and implementation of humane endpoints, and sharing good practice with other practitioners, regulators and the public.

The RSPCA's Focus on Severe Suffering project has produced resources that facilitate all of these requirements, contributing to a 61 % reduction in experimental procedures causing severe suffering in the UK since actual severity data began to be collected in 2014 [2,3]. These include a practical "Roadmap" exercise to help establishments focus on procedures that have the potential to cause "severe" suffering, identify contributing factors and find ways of avoiding or refining these.

The approach is based on an "audit" of procedures, carried out by an appropriate team of people with different expertise and perspectives, including animal technologists and care staff, scientists and the attending veterinarian. This enables comprehensive analysis of the lifetime experience of each animal, so that experimental harms and other stressful experiences can be identified and refined. The aim is to apply the principle of "marginal gains" so that severe suffering can be reduced or, ideally, avoided.

This presentation will explain the Roadmap, and how this can be used to refine inherently severe procedures, identify and address cumulative severity, and avoid mortality. The Roadmap is freely available online, and has been designed to tie in with current European Commission guidelines and requirements of the Directive, to maximize benefits without requiring significant additional resources.

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Submission declaration:
Conflicts of interest: The author declares that there are no conflicts of interest.
Statement on ethics vote: Not applicable.

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A dynamic *in vitro* model of smooth muscle and endothelial cell cultures for vascular calcification study

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Vascular calcifications (VC) are abnormal mineral depositions in the vascular tree and are frequently observed in patients with atherosclerosis, chronic kidney disease (CKD), and diabetes [1,2]. VC affects more than 70% of the population over 70 years, leading to different cardiovascular complications, thus increasing morbidity and mortality. Currently, medical treatments are not available; preventive actions based on diet and integration with natural bioproducts, like quercetin, curcumin, resveratrol, and magnesium, have been reported to inhibit the disease process in experimental models [3]. An in-depth understanding of the molecular mechanisms of vascular calcification represents the key to developing effective therapeutic strategies to treat VC. A valuable approach for better understanding underlying mechanisms is represented by *in-vitro* cell model systems that recapitulate the cellular characteristics and their hemodynamic environment. In particular, these systems may allow to evaluate also the role of active compounds in the treatment of vascular calcification. This work aims to develop an advanced *in-vitro* model that can recapitulate the complex biological environment of the vascular wall to increase the effectiveness of *in-vitro* tests of active compounds in the reduction and/or slow down of vascular calcification before proceeding to clinical studies.

To best mimic the environment of systemic vasculature, Human Coronary Artery Endothelial cells (HCAECs) and Human Coronary Artery Smooth Muscle cells (HCASMCs) were tested using a double-flow bioreactor (LiveBox2, IVTech Srl, Massarosa, Italy) composed by two chambers that can be perfused by independent flows thus allowing static and/or dynamic conditions. HCAECs (45,000 cells) were seeded in each upper chamber of the LiveBox2 and cultured for seven days in supplemented Endothelial Cell GM MW2. HCASMCs (30,000 cells) were seeded in each bottom chamber of the LB2 and cultured in supplemented Medium 231; the two compartments are separated by a polyester porous membrane (0.45 μm pore size). Upper chambers were subjected to a 250 μL/min flow rate for the dynamic condition. For the induction of HCASMCs calcification, Medium 231 was replaced with DMEM HG supplemented with 1.9 mM phosphates solution (NaH_2PO_4/Na_2HPO_4) for seven days. After the incubation period, HCAECs and HCASMCs viability was tested with CellTiter-Blue Cell Viability Fluorometric Assay. For Calcium quantification, HCASMCs were subjected to HCl and overnight freezing to optimize the lysis, and Sigma-Aldrich Calcium Colorimetric Assay Kit was used following manufacturer instructions. Cell samples were collected, and Real Time-PCR of specific genes was performed. Data analysis was conducted using the Student's t-test or ANOVA with a significance level of $\alpha = 0.05$.

The analysis of cell viability demonstrated that calcifying media significantly decreased HCASMCs viability in static conditions. The presence of HCAECs and the dynamic flow seem to attenuate the antiproliferative effect of the phosphate solution on the HCASMCs. Regarding HCAECs' viability, we found no significant differences during calcifying conditions with respect to control, both in static and dynamic conditions. The positive effect of HCAECs and the dynamic flow was also highlighted in calcium amount, which is considerably reduced for static conditions and when endothelial cells are present. To assess the ability of phosphates mixture to induce the osteoblastic switch of HCASMCs, a key event in vascular calcification, the Real Time-PCR was performed and showed, with respect to control, a significant increase of: 1) BMP2 and BMPR1a receptor, as well as of BMPR1b, also if no significantly involved in vascular calcification; 2) IL-1β, a potent proinflammatory cytokine playing diverse roles in the pathogenesis of atherosclerotic vascular calcification; 3) CX43, the most abundant gap junction protein involved in osteoblastic differentiation; 4) mTOR1, which is involved in the maintenance of cellular homeostasis.

This work was supported by SOFIA Project Co-Funded by Regione Toscana within Programma operativo regionale (Por) Fondo europeo di sviluppo regionale (Fesr) 2014-2020 – Bando 2: Progetti di ricerca e sviluppo delle MPMI.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Anti-hyperglycemic, anti-hyperlipidemic, anti-inflammatory and antioxidant activity of Amachol®, an aqueous amaranth seed extract, using a human small intestinal organotypic culture model

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In these years, there is a growing interest towards plant raw materials with potential health benefits since they can be used as ingredients in food preparations. The aim is to produce functional foods whose intake is claimed to promote a good state of health, thereby preventing diseases in consumers. In this regard, pseudo cereals, such as amaranth, are gaining interest as beneficial ingredients for food production. Amaranth is the source of a wide spectrum of biologically active compounds with valuable health-promoting effects. Among the different amaranth species, *Amaranthus cruentus*, *A. hypochondriacus* and *A. caudatus* are grown in many countries for food purposes. From a nutritional point of view, the amaranth seeds are a rich source of lipids and proteins used for flour, flakes, bread and pasta production [1]. Several *in vivo* studies have demonstrated the beneficial effects of amaranth seed flour diet supplementation with antioxidant and hypolipidemic activities and of oil diet supplementation with hepatoprotective, hypoglycemic and blood pressure lowering properties, respectively [2,3].

The present study aimed to analyze the beneficial properties of Amachol®, an aqueous extract from *A. caudatus* seeds, investigated by the sole use of human-relevant *in vitro* models. Amachol® was initially subjected to a simulated gastro-intestinal digestion. Thus, a human small intestinal organotypic culture model, namely EpiIntestinal™, was employed to mimic the intestinal absorbance of the digested Amachol® extract in presence of glucose or of a cholesterol analogue to highlight its hypocholesterolemic and hypoglycemic properties, as well as the ability to deliver polyphenols into the bloodstream. Human pancreatic islets were employed to measure the amount of insulin release stimulated by the EpiIntestinal™ tissue absorbed glucose in presence or absence of the amaranth extract. Furthermore, the hypolipidemic properties of Amachol® were investigated in human Adipose Tissue Mesenchymal Stem Cells (hATMSCs)-derived adipocytes, while the anti-inflammatory and antioxidant properties were studied in human peripheral blood mononuclear cells (PBMCs) as well as in human M0 macrophages differentiated from the THP-1 monocytic cell line.

Our results show that both glucose as well as cholesterol intestinal absorption and transport into the basolateral compartment of the EpiIntestinal™ tissue, in concentrations mimicking a full meal, were significantly inhibited by the concomitant administration of the digested Amachol® extract. In addition, the extract itself was able to promote the intestinal absorption of significant amounts of polyphenols whose bioactive properties are very well known. Pancreatic islet treated with EpiIntestinal™ basolateral media deriving from glucose and Amachol® co-absorption experiments showed a significantly reduced insulin release compared to those deriving from the EpiIntestinal™ tissue incubated with glucose alone. Furthermore, hATMSC-derived adipocytes showed a lower lipid accumulation in response to glucose administration, in presence of the Amachol® extract, as well as a lower differentiation potential towards mature adipocytes, indicating the ability of the extract to affect the lipid storage and metabolism in the human adipose tissue. Finally, it was also possible to highlight an anti-inflammatory and antioxidant activity of the extract in PHA-induced PBMC proliferation, in LPS-induced macrophage activation and in H_2O_2-stimulated macrophage ROS production *in vitro* cellular models, respectively.

Overall, we demonstrated that by use of human *in vitro* models, although simplistic in comparison to an *in vivo* setting, it is possible to measure relevant nutraceutical and pharmacological properties of functional foods through a process mimicking the gastro-intestinal digestion and absorption, approaching the overcoming of the animal models for such studies.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Evaluation of cytotoxicity and pro-inflammatory effects of Russian chrysotile fibers on the EpiAirway *in vitro* human airway model

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The term asbestos is used to collectively refer to six naturally – occurring fibrous minerals, i.e., crocidolite, amosite, tremolite, actinolite, anthophyllite and chrysotile, which have been exploited in numerous commercial and industrial settings for many years. Since there is evidence of the correlation between occupational and non-occupational asbestos exposure and the onset of diseases such as asbestosis, pleural effusion, pleural plaques and diffuse fibrosis, lung cancer and pleural mesothelioma, the International Agency for Research on Cancer (IARC) has classified the asbestos fibers as carcinogenic to humans [1]. In this regard, the sale and the use of all asbestos fibers in more than 50 countries is currently banned. However, China, India, Kazakhstan and Russia continue to produce, utilize and export chrysotile because, according to them, its low biodurability renders it less dangerous to human health than other asbestos fibers. Therefore, the scientific debate on the potential harmful effects of chrysotile, and consequently on its use, is still a very open question [2,3].

Starting from these differing opinions, this study is aimed at evaluating the early effects of the commercially-available Russian chrysotile [4] on a 3D-*in vitro* lung tissue model (EpiAirway™, MatTek Corporation) consisting of normal, human-derived tracheal/bronchial epithelial cells maintained in an Air-Liquid Interface (ALI).

The tissues were exposed to 100 µg/mL of the Russian Chrysotile fibers (at two different lengths < 5 µm [CHR-S] and > 5 µm [CHR-L]) and of UICC Standard Crocidolite (CRO) fibers (positive control) for 24 and 48 h. At the end of each experimental time the tissue viability was evaluated by MTT and the profiles of IL-1β, TNF-α, IL-6 and IL-8 pro-inflammatory cytokines were analyzed in terms of gene expression (qPCR) and protein release in the tissue media (MILLIPLEX® assay).

Although all the tested mineral fibers significantly decreased the tissue viability at both time frames compared to the untreated tissues, it is noteworthy that, after 24 h, both CHR-S and CHR-L induced a more marked and early cytotoxic effect compared to CRO while, at 48 h, CRO reduced the tissue viability to the same extent as both of the CHRs. After 24 h of exposure, both the CHRs and CRO increased the gene levels of IL-1β, IL-6 and IL-8 pro-inflammatory cytokines. Moreover, MILLIPLEX® assay evidenced a release of TNFα, IL-1β and IL-8 into the tissue media after 24 and 48 h exposure to the CHRs and CRO, with CHR S releasing a greater amount of IL-6 than CHR L. This preliminary toxicity study on the biological effects of the two different length sizes of the Russian Chrysotile proved that both are able to induce an early decrease of the MTT viability index, triggering the activation of pro-inflammatory signals. Currently, CHR, despite being considered less harmful among asbestos fibers, have behaved in a similar way to CRO. In fact, since the low biodurability of CHR results in a faster release of ions (i.e., Fe, Cr, Co, Mn, Ni and Cu), it may represent one of the factors involved in cell damage, probably via oxidative stress [5].

In conclusion, this study has highlighted that Russian CHR can exert the same cytotoxic mechanisms as amphiboles, inducing pro-inflammatory pathways in the lungs that can lead to adverse chronic pathological conditions. Therefore, it is necessary to shed more light on these fibers in terms of their harmful potential and their long-term effects on human health.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Optimization of skin sensitization testing strategy *in vitro* for medical device extracts

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Before marketing, medical devices have to be tested in accordance with ISO 10993-10 to avoid skin sensitization. This standard predominantly refers to *in vivo* tests, however, it doesn't exclude the use of alternative *in vitro* methods, which have been sufficiently technically and scientifically validated [1]. It is foreseen that due to the complexity of the sensitization endpoint, combination of several methods will be needed to address all key events of the skin sensitization Adverse Outcome Pathway. The objective of this study was to evaluate the sensitization potential of 97 commercially available samples of medical devices using a combination of *in vivo* (LLNA DA, OECD TG 442A), *in chemico* (DPRA, OECD TG 442C) and *in vitro* (LuSens, OECD TG 442D) methods with the aim to enhance the testing strategy for safety assessment of medical device extracts, to optimize the test and extraction procedures and to extend the applicability domains of separate *in vitro* methods recently successfully validated for chemicals. A good agreement between *in vitro* and *in vivo* results was achieved regarding the absence of skin sensitization potential; however, discrepancies in positive classifications have been recorded. The mismatch between *in vitro* and *in vivo* results might be caused by specific response of the immune system of the living organism, however, the *in vitro* methods are suggested as feasible for bottom-up skin sensitization testing, starting with test methods accurately identifying non-sensitizing medical device extracts [2].

Supported by ERDF/ESF project "International competitiveness of NIPH in research, development and education in alternative toxicological methods" (No. CZ.02.1.01/0.0/0.0/16_019/0000860).

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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An optogenetic approach to characterize human TLR2 homo- and heterodimers in THP-1 and THP-1 derived macrophages

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Toll-like receptors (TLRs), one of the best known pattern recognition receptors, are key players in infectious diseases and complications as sepsis [1].To date, little is known about the functional mechanisms of the TLR2 homodimer [2]. Mainly the heterodimers with TLR1 and TLR6 which show pro-inflammatory properties are discussed. The scientific body is not in agreement about the role of TLR2 homodimers, especially since the specific ligands for activation are unknown [3,4]. Furthermore, the overall role of TLR2 in adhesion and migration during an inflammatory event is still not fully elucidated [5]. However, a profound understanding may be particularly valuable in immunological dysfunctions as in sepsis.

To gain more clarity on the role of TLR2, we have established a novel optogenetic cell line using THP-1 TLR2 knock-out (KO) cells. For this purpose, human TLR2 was fused with a light-oxygen-voltage-sensing (LOV) domain isolated from *Vaucheria frigida aureochrome 1* which allows precise dimerization and activation of TLR2 by blue light. The TLR2LOV construct was then stably integrated into THP-1 and THP-1 KO cells by lentiviral transfection. We performed Western Blots and reporter assays to ensure the presence and functionality of the optogenetic cell lines. To examine mRNA expression levels of common pro- and anti-inflammatory cytokines and chemokines (IL-6, IL-8, IL-10, TGFβ) we conducted RT-qPCR. Moreover, we have established adhesion, attachment and migration assays and differentiated THP-1, KO and TLR2LOV knock-in cells into macrophages.

By using an integrated cell-reporter we were able to show the functionality and effective activation of our optogenetic TLR2LOV knock-in cell line. Preliminary data demonstrate significant differences in mRNA and protein expression levels between KO and TLR2LOV knock-in cells after light induction and a difference between homo- and heterodimers. In addition, KO cells showed stronger attachment which could be rescued by the knock-in cells. Macrophage differentiation was more prominent in KO cells than in THP-1 and TLR2LOV knock-in cells.

In conclusion our optogenetic cell lines are a convenient tool to further investigate principal mechanisms of TLR2 homo, -and heterodimerization and may provide new insights for therapeutic strategies for sepsis and other immunological disorders.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Knowledge and attitude towards laboratory animal use and 3Rs: A cross-sectional study in Sudan

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Background: Although animal experimentation is common in Africa, national and institutional policies, and guidelines on using animals in research are limited [1]. Unfortunately, Sudan's status is not an exception, as professional training is limited in both undergraduate and postgraduate levels. This study aims to assess the knowledge and attitude of undergraduate and postgraduate pharmacy students towards laboratory animals and the ethical issues concerning animal use in pharmacy research to provide authorities with information that could facilitate successful intervention.

Method: A total of 59 respondents completed an online semi-structured questionnaire. The study survey was conducted from November 2021 until December 2021. We compared the knowledge about laboratory animals among 5th-year pharmacy students, bachelor's degree holders, and postgraduate students. We assessed factors associated with higher knowledge of laboratory animals. Moreover, we examined respondents' rationale for classifying laboratory animals from the most ethically acceptable to the least ethically acceptable animals. We used one-way analysis of variance (ANOVA) and Chi-square test (with Fisher's exact test) to compare variables between groups in the sample. Ethical approval was obtained from the Ethics Committee of National University (No. NU-REC/08-021/02)

Results: 94.9% of the respondents had worked directly on laboratory animals. However, only 50.8 % of them had a training on laboratory animals. Regarding the participants' knowledge of laboratory animals, no difference was observed between undergraduate and postgraduate students ($p = 0.216$). 37.3% of respondents classified the dog as the least ethically acceptable laboratory animal, while 28.8% chose the Rhesus monkey. Similarly, 55.9 % of respondents classified the rat as more ethically acceptable than Drosophila melanogaster, indicating a lack of proper knowledge. Regarding the ethics of laboratory animal use, 16.9% of the participant were able to name the 3Rs (Replacement, Reduction and Refinement). In the given hypothetical experiment scenario, 59.3% of respondents prioritized refinement while only 15.3% prioritized reduction. Moreover, 49.1% did not know where to submit the ethical permission application. Finally, 67.8% of respondents were willing to participate in laboratory animal training courses if offered.

Conclusion: In this pilot study, the undergraduate and postgraduate pharmacy students in Sudan lack proper knowledge or training about laboratory animal use and ethics issues concerning them. The findings could be a valuable future reference assess the knowledge gap and take a proper decision by authorities in teaching and research institutions towards laboratory animal use in Sudan.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Not applicable.

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Antibodies: Resistances to overcome on the way to a cruelty-free future

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Although efficient methods for the animal-free production of antibodies are available, it is estimated that more than 1 million animals are used for the production of antibodies every year in the EU [1]. This contradicts the EU directive 2010/63/EU, which does not permit the use of animals if other, animal-free methods are available [2]. In response to this situation, the EU reference laboratory for alternatives to animal testing (EURL ECVAM) issued a recommendation against the further use of animals for the generation and production of antibodies [1].

The most advanced method for developing non-animal antibodies is phage display technology [3]. Here, the genetic information for the production of antibodies is introduced into phages, and from the obtained phage libraries antibodies can be selected in an *in-vitro* process. This has recently been awarded by the European Coalition to End Animal Experiments (ECEAE) with the first prize dedicated to the development and application of animal-free antibodies. Among other advantages, phage display allows for the straight-forward development of fully human antibodies and to optimize the antibodies specificity during the selection process.

In particular in the therapeutic area a number of phage display-derived antibodies have already been approved and many more are in clinical trials [4] A prominent example for a phage display-derived antibody is Humira (adalimumab), a drug used to treat rheumatoid arthritis and other inflammatory diseases, that generated sales of more than 20 billion US dollars in 2021. While the phage display method is already established in antibody development for therapeutic applications and first products entered the market very successfully, there is still considerable resistance to phasing out the development and use of animal-based antibodies, especially in the biomedical research community.

We thus analyze a whole series of statements published by various scientific and industrial organizations in response to the ECVAM recommendation on non-animal-derived antibodies. We subject the stated shortcomings of animal-free antibodies to a fact check and discuss how the paradigm change from animal-derived to animal-free antibodies can succeed.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote is required.

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Designing 3D bioengineered *in vitro* cardiac tissue models as reliable tools for the evaluation of chemical cardiotoxicity

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Humans are continuously exposed to a huge amount and a variety of chemicals. Animal tests are the gold standard for toxicity testing. However, they often fail in finely replicating the real physio-pathological scenario, and their use is associated with ethical issues. Thus, there is an urgent need for Novel Approach Methodologies providing more reliable and robust methods for toxicity assessment. In this regard, 3D *in vitro* tissue models more efficiently mimic the native human environment and bring clear ethical advantages [1]. To design a 3D bioengineered tissue model, the 3D matrix used to guide cell behavior, extracellular matrix (ECM) production, and new tissue formation should replicate the architecture and composition of the native tissue. In this context, the proper selection of the biomaterial, the fabrication method, and the functionalization protocol is pivotal. In cardiac tissue engineering, elastomeric polymers are required as constituents of porous structs replicating *in vitro* myocardium architecture and mechanical properties. Moreover, surface functionalization with cardiac ECM proteins can be exploited to replicate *in vitro* the biochemical cues present in the native tissue. In this work, we exploited the versatility of poly(urethane) (PU) chemistry to design a plethora of polymers with a wide range of physico-chemical properties with the final aim to identify the most suitable one to be used to fabricate a 3D framework for *in vitro* cardiac tissue model development. PUs were synthesized using poly(ε-caprolactone) diol (2000 Da), an aliphatic diisocyanate, and different chain extenders (e.g., 1,4-butanediol, 1,8-octanediol, L-lysine ethyl ester, N-Boc serinol) [2]. PU physico-chemical properties were thoroughly characterized, with particular attention to their mechanical performances. In detail, 1,8-octanediol gave a PU with higher elongation at break (ε%, ≈ 30-40%), while 1,4-butanediol resulted in a more brittle and stiffer PU. Conversely, N-Boc serinol gave a PU with around 150 MPa Young's Modulus (E) and ε% of approx. 150%. L-lysine ethyl ester instead provided the resulting PU with an elastomeric behavior (E and ε% of around 10 MPa and 700%, respectively) that made it the optimal one to fabricate constructs replicating the cardiac ECM. This PU was then microfabricated by thermally induced phase separation and melt extrusion additive manufacturing, resulting in porous structs with aligned pores along a preferred direction and multi-layered matrices with a 0°/90° lay-down pattern, respectively. The structs were surface plasma treated in the presence of acrylic acid to expose -COOH groups and then grafted with ECM proteins (laminin, LN, fibronectin, FN) through carbodiimide chemistry [3,4]. Colorimetric assays and spectroscopic analyses proved the successful functionalization of the constructs. Lastly, the developed *in vitro* replicas of the cardiac ECM were seeded with cardiac cells (rat neonatal cardiomyocytes, CMs, cardiac progenitor cells, CPCs) to establish cardiac tissue models. LN promoted CPC proliferation and expression of differentiation markers for CMs, endothelial, and smooth muscle cells. CMs exhibited a high survival rate and stable beating. RT-PCR evidenced a relevant modulation of cardiac muscle, hypertrophy-specific, and metabolism-related genes at 14 days of cell culture. Our results proved the potential of the developed structs as cardiac tissue models with tunable structural, mechanical, and biochemical features. Such models will allow the investigation of physio-pathological processes and cardiotoxicity testing.

This work was supported by MIUR-FIRB 2010 Future in Research (RBFR10L0GK), MIUR-PRIN 2010-2011 (2010J8RYS7), and the European Union's Horizon 2020 research and innovation program (101037090). The content of this publication reflects only the authors' view, and the Commission is not responsible for any use that may be made of the information it contains.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

65

Transition to non-animal science – Current state of play in the EU

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The European Commission and some European countries have, throughout the last years, declared intentions to reduce and replace the use of animals in science. The new German government is committed to implement a reduction strategy for animal experiments and promote research on alternatives, their implementation and a cross-sectoral competence network [1]. The Swedish 3R Center has published a preliminary strategy to further limit the use of animals in science and is continuing this work in 2022, focusing on further discussions with stakeholders on content and actions [2]. The Netherlands has an initiative in place to transition to non-animal science, where cooperation between government, society, industry and science is key to define together the optimal direction for the intended change. The Norwegian National Experimental Animals Committee also proposed, in an August 2020 report, steps to develop a concrete plan for a transition to non-animal science [3]. At EU level, the Directive 2010/63/EU on the protection of animals used for scientific purposes sets out a clear objective to fully replace the use of animals in science.

However, 2020 reports of the European Commission revealed that more than 23 million animals were impacted by scientific practices in the EU in only one year. Almost 60% of these animals are not regularly accounted for, as they are killed in EU laboratories without actually being used in experiments. While the use of non-animal methods grows in some areas like safety testing of chemicals and drug development, recent decades have also seen increases in the number of genetically altered animals. 43% of all animals impacted by science in 2017 in the EU were associated with the creation, maintenance or use of genetically altered animals. Besides the wide use of mice and zebrafish, non-human primates have also been genetically altered in recent experiments.

In September 2021, the European Parliament adopted a resolution calling on the European Commission to establish an EU-wide Action Plan to transition to non-animal science [4]. The European Parliament recognised that an active, coordinated approach is necessary to achieve sustainable reductions and, ultimately, the full replacement of animals used in science. The Members of the European Parliament (MEPs) also ask for targeted funding across all EU research and innovation initiatives, and adequate training for non-animal methods in all relevant courses. This resolution was supported by an overwhelming majority of MEPs.

Just before the vote in the European Parliament, a number of scientists showed their support for the resolution to facilitate the transition to non-animal science through the definition of common priorities, allocation of funding and multidisciplinary and inter-service collaborations; but the European Commission remains stagnant. Six months after the resolution of the European Parliament, the European Commission responded with a list of fragmented initiatives that could eventually lead to some reduction in the use of animals, but without committing to steps to implement the requested action plan to transition to non-animal science. Moreover, as part of the Chemicals Strategy for Sustainability ambitions to achieve a "toxic-free environment" by 2050, the European Commission is revising EU chemicals legislations. Under these actions, the Commission implies that reducing and replacing traditional animal testing with non-animal testing methods will weaken the protection of human health and the environment, awaking criticism from the scientific community [5].

Traditional reliance on animal-based research and testing is still strongly rooted within regulations and in scientific norms, but the possibilities for better, more predictive and humane science are growing. The complex processes behind a transition involve multiple layers of values, knowledge and society and, at this particular moment, an appropriate governance model is needed to facilitate the shift towards non-animal science.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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

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The use of laboratory animals is increasingly criticized in societal, political and scientific debates. In scientific debates, there is increasing concern on the predictability of animal studies for humans. In societal and political debates, a shift in animal-human relations can be observed over the last decades in which our relationship with animals is being seen less from an instrumental perspective, influencing debates on animal testing [1].

In this presentation, which brings together insights from several recent publications, I argue that a transformative governance approach is needed to accelerate the transition to animal-free innovation. I thereby approach animal-free innovation from a transition and transformative governance perspective. The transition literature is often used to analyze sustainable development, including the energy transition, transition to a circular economy, food transition including the protein transition, and the transition to animal-free innovation. In this presentation, the concept of sustainable development is seen to include environmental issues, justice and animal interests, in other words – ecocentric, compassionate and just sustainable development [2]. The multi-level perspective on transitions [3] theorizes that changes in a regime are enabled by niche innovations (new technological and/or social innovations). Regimes also interact with the so-called landscape, which represents larger societal developments and trends (e.g., globalization, societal values).

The transition literature is part of a larger body of literature on fundamental societal changes. While a significant body of literature studies these fundamental changes, less thought has gone into how to govern (or steer) them [4]. Such transformative governance includes five governance approaches, namely: integrative, inclusive, adaptive, transdisciplinary, and anticipatory governance. Governance can only become transformative when the five governance approaches are: a) focused on addressing the underlying causes of sustainability issues; b) implemented in conjunction; and c) operationalized in a specific manner [2]. The presentation will show how a transformative governance approach can support efforts by different stakeholders to accelerate the transition to animal-free innovation.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Not applicable.

68

Cross-species-relevance of *in vitro* bioassay battery for the assessment of thyroid hormone disruption

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The applicability of data obtained with the alternative *in vitro* bioassays in risk assessment is often hindered by the lack of complexity of the *in vitro* assays. However, the applicability could be significantly improved by the application of the Adverse Outcome Pathway (AOP) concept that interlinks the molecular initiating events (MIEs), assessed *in vitro*, with the adverse outcome (AO) on individual or even population level [1]. The EU H2020 ERGO project is using this concept to design a battery of *in vitro* bioassays for screening chemicals for endocrine-disrupting properties on the thyroid hormone (TH) system that is highly conserved across vertebrates, which allows to obtain cross-species relevant data [2]. Cross-species AOP network for thyroid hormone disruption is being developed and serves as a basis for prioritization of the MIEs to be addressed in the bioassay battery.

We have established and optimized a set of bioassays focusing on prioritized endpoints in thyroid hormone disruption. The cross-species relevance of the selected MIEs has been supported by a comparison of the primary structure of target proteins across animals using an online tool Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS) from US EPA [3]. The assays for the assessment of effect of TDCs on iodide uptake by thyroid cells mediated by Na^+/I^- symporter (NIS) are based on stably transfected human cell line overexpressing NIS and on rat thyroid cell model, with the detection of uptaken iodide levels using a non-radioactive colorimetric Sandell-Kolthoff reaction. The results document that the NIS expression level has a significant impact on the detected IC_{50} levels of the model inhibitors. Transport of TH is addressed by fluorometric assessment of displacement of TH from its plasma transporter transthyretin (TTR) by TDCs. The TH metabolization step is addressed by a cell model with a reporter gene under transcription control by aryl hydrocarbon receptor (AhR) that plays a significant role in triggering the metabolization systems. Interaction with thyroid receptor signaling is assessed using reporter-gene assay. These assays have been established and optimized for screening of a set of prioritized chemicals and environmental samples from the aquatic environment for their potential to disrupt the thyroid hormone system. The results demonstrate the utility of the newly developed bioassay battery for high-throughput screening of chemicals as well as environmentally relevant complex pollutant mixtures for the characterization of their thyroid hormone-disrupting potential and identification of the most relevant MIEs. Based on SeqAPASS analysis, the data from the established human cell-based bioassays should be relevant to most vertebrate species.

The project has received funding from the EU H2020 research and innovation programme under grant agreement No. 825753 and Czech Science Foundation (GACR) under grant agreement GX20-04676X.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Evaluation of a new microparticulate depot formulation of buprenorphine for sustained post-surgical analgesia

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Adequate pain management is essential for ethical and scientific reasons in animal experiments. The selection of analgesics in fracture models is restricted due to potential interfering properties of anti-inflammatory drugs during the initial and inflammatory phase of fracture healing. Current depot/sustained-release formulations of Buprenorphine – a potent analgesic in pre-clinical studies – are only available in the US and several attempts to import these products to Europe have failed. Schreiner et al. recently published a study on the successful development and use of a newly developed poly-lactic-co-glycolic acid (PLGA) based microparticulate Buprenorphine formulation for sustained drug release to provide an alternative product within Europe [1]. Here, we investigated whether the administration of the newly developed sustained-release Buprenorphine formulation (BUP-Depot) ensures continuous and sufficient analgesia in mouse femoral fracture models as a potent alternative to the application of Tramadol via drinking water.

To include several aspects such as fixation stiffness (rigid vs. flexible) and sex (female vs. male) – 8 experimental groups (n = 10 per group) were investigated in parallel, comparing post-surgical analgesia with the newly developed sustained-release Buprenorphine (BUP-Depot) and the common application of Tramadol for 1 day prior and 3 days post-surgical. The study was approved by the local authority – (LaGeSo; permit numbers: G0044/20), performed in accordance with the German Animal Welfare Act and preregistered in the Animal Study Registry. A total of 80 C57BL/6N female and male mice (Charles River) aged 8 weeks were housed in pairs. Cages were enriched and all mice were tunnel handled. Mice underwent surgery at an age of 12 weeks as published previously [2]. At the end of the surgery, respective mice received the BUP-Depot (s.c. 1.25 mg/kg). The other group received Tramadol (0.1 mg/mL) via the drinking water starting 24 h before surgery until day 3 post-surgical. We monitored (i) general parameters of wellbeing, e.g., body weight, water and food intake, and (ii) model-specific pain parameters including walking behavior and CatWalk analysis. Mice were euthanized at 14 d post-surgery and fractured femora were cryo-embedded for further analysis.

The body weight and food intake were decreased at 24 h and 48 h after surgery regardless of sex, analgesic regime, and fixation and reached the initial levels at 72 h in all groups. We did not find differences in water intake in the groups receiving Tramadol via the drinking water when compared to the BUP-Depot groups. Clinical assessment using a composite score (facial expression and overall appearance) indicated a significant higher composite pain score in male mice of the flexible fixation group treated with Tramadol at 24 h and 48 h after osteotomy when compared with the other groups. With respect to model-specific behavior, limping was partially observable in all groups for up to 72 h post-surgical with no differences between groups. Moreover, decreased rear-up behavior with no differences between treatment groups was observed. Additional CatWalk analysis was used to specifically delineate alterations in locomotion in the fractured limb with no differences with regard to the analgesia or fixation, except for male mice with flexible fixation and Tramadol treatment, which showed reduced velocity and altered gait patterns. Histological analyses of the femora indicated clear differences between the fixation methods and sexes but no obvious influence of the analgesic protocol.

Our data provide evidence that the administration of the newly developed sustained-release Buprenorphine formulation (BUP-Depot) ensures continuous and sufficient analgesia in mouse femoral fracture models and therefore serves as a potent alternative to the application of Tramadol via drinking water. The availability of a sustained-release formulation of Buprenorphine in Europe would be imperative for pain management in preclinical studies to increase animal welfare and actively support the implementation of the 3R principle (Refinement).

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Evidence-based transition to animal-free innovations: Let's make it happen!

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The results from many preclinical systematic reviews have revealed that publication quality from animal studies is insufficient and is hardly improving over time, despite the wide acceptance of the ARRIVE guidelines for reporting. Moreover, evidence reveals that many animal studies do not translate well to the human situation [1,2]. For example, many adverse drug reactions occur in humans, despite the fact that drugs were considered safe for use in humans based on animal study results [2]. In a historical analysis we discovered that the evidence base for legally required animal studies turned out to be at least very uncertain, and that (new) requirements for animal studies in legislation were seen to be taken as a response to crises [3]. The Covid crisis in turn, seems to have led to a reversed situation. We analyzed the case study of the marketing approval for the Pfizer/BioNTech mRNA vaccine. There was a much faster vaccine conditional marketing approval – in 10 months instead of 10 years – by allowing fewer animal studies and more alternatives in the regulatory process [4]: only "essential" animal studies were required, animal studies ran in parallel – instead of sequentially – with clinical trials, historical results from earlier vaccines were also accepted and batch releases were performed with alternative tests only, which was made possible due to the thorough characterization of the mRNA vaccine.

The evidence that new approach methods lead to better translation is increasing, and as these new methodologies will lead to faster, better, more ethical and ultimately cheaper developmental chains as compared to animal studies, it is high time to investigate how these new methods can become widely accepted and implemented in practice soon. This needs interdisciplinary transition research in how this can be accomplished in the best possible way as fast as possible [5], as it needs further validation of new methodologies and processes (niche level), new legislative guidelines (regime level) and societal/political changes (landscape level). It is also essential to involve all key stakeholders in these processes, to be able to implement the desired changes in practice fast and effectively.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest.
Statement on ethics vote: Not applicable.

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The role of the animal welfare body in ensuring compliance with the 3Rs: A case study of the forced swim test

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Directive 2010/63/EU requires that an animal welfare body advises users of animals in scientific procedures on the requirements for replacement, reduction and refinement (3Rs) (Article 26; Article 27). One of the responsibilities of an animal welfare body is to keep "informed of technical and scientific developments" that contribute to the application of the 3Rs (Article 27). The animal welfare body typically provides guidance to ensure that project license applications are of sufficient quality to be submitted and approved by the national competent authority; overseeing, reviewing and approving (at a local level) research involving animals, including critically evaluating projects for any ethical and welfare issues, and ensuring a commitment to good quality science. However, across the EU and UK, there appears to be considerable inconsistency between the guidance provided by animal welfare bodies. This is concerning for both animal welfare and quality of scientific output. Using the Forced swim test (FST) as a case study, we discuss factors that animal welfare bodies should consider to ensure compliance with Directive 2010/63/EU and consistency across member states.

The FST is a behavioral experiment largely used for modelling depression-like behavior and for screening antidepressant drugs. The test measures an animal's latency to become immobile in an inescapable beaker filled with water, with no rest platform. Historically this behavior has been interpreted as "behavioral despair". However, the test has been widely criticized for its validity, and it has been argued that immobility may not be a sign of despair, but rather a positive sign of learning, conserving energy, and adapting to a new environment [1]. An influential paper was published in 2021, comprising authors from the Medicines and Healthcare products Regulatory Agency (MHRA), the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), and King's College London (KCL), and that was initiated by the Animals in Science Regulation Unit at the UK Home Office. This publication calls on researchers to seek alternatives to the use of the FST for antidepressant drugs, noting that the test could rule out the discovery of effective new drugs for humans, and recognizing that it is a poor model for human depression [2]. Later in the same year, a review highlighted the test as poor in accurately identifying antidepressants for human use [3].

Use of the test has also been discouraged on animal welfare grounds, with the NC3Rs identifying the test as "highly contentious" and "under considerable scrutiny" due to "its severity and effect on animal welfare" [4]. By its nature, the test is stressful. In the UK, the FST is usually categorized as "moderate" severity due to its impact on animal welfare. However, there has been at least one reporting of its classification as "mild", arguably erroneously, because of apparent refinements implemented. There are also cases from EU member states where severity classification has been inconsistent, for example: in the Netherlands the procedure was assigned as "severe" for mice and "moderate" for rats; and in Germany the procedure was assigned as "non-recovery".

A number of pharmaceutical companies and academic institutions have policies that prevent the FST being carried out at their establishment, however, the test is common in both basic research and in testing. Peer reviewed publications continue to illustrate wide use of the test across member states, which state that the procedures have been approved by local animal welfare bodies, where authors claim that immobility is directly proportional to depression-like behavior. Similarly, in the UK, despite the prominent publication from the MHRA, NC3Rs and KCL, animal welfare bodies and the national competent authority continue approve project license applications where procedures include this test.

Here we highlight the evidence for ending use of the FST in research for depression and testing of antidepressants based on scientific and welfare grounds and identify other areas of its use where welfare is compromised. We propose that animal welfare bodies have the responsibility to advise against the use of the test at their establishment, in compliance with the EU and national legislative requirements for the 3Rs and must encourage the use of more valid methods for human mental health research.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Prediction of human toxicology with alternative experimental models – Zebrafish embryo (EU project PrecisionTox)

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Major approaches to replace classic animal testing for human toxicity are based on predictions using cellular *in vitro* or computational models. The EU project PrecisionTox aims at using a suite of non-sentinel alternative model organisms for human toxicology prediction – zebrafish embryo, xenopus embryo, daphnia, nematodes, drosophila, together with human cell lines. The approach assumes that the phylogenetically distant model organisms share common toxicity pathways and/or together might capture pathways of high relevance for human toxicity. In the frame of the EU project PrecisionTox we focus on the zebrafish embryo model which represents a valid alternative to standard mammalian testing and aligns with the 3Rs concept. Furthermore, it supports high content chemical screening to characterize thousands of chemicals in commerce with missing toxicological information. As a complex *in vivo* model, the zebrafish embryo test (FET) enables to understand toxicological mechanisms that are difficult to assess in cellular models. For instance, the zebrafish embryo test allows the high-throughput assessment of phenotypic changes as well as motor behavior and heart function which are important and relevant endpoints in toxicology. Therefore, the main goal of the present study is to improve a previously developed semi-automated high content workflow [1] to assess the effects of 250 chemicals on several sublethal endpoints using automated imaging and behavior tracking in zebrafish embryos. Image feature extraction is supported by machine learning. Additionally, automated KNIME/R-based workflows allow the high throughput extraction of concentration-response curves and effect concentrations. Our testing approach represents a major modification of the procedure described in the OECD TG 236 [2]. While the OECD 236 guideline focuses on acute toxicity with particular application in ecotoxicology, our workflow is a further miniaturization and includes additional toxicological endpoints for the assessment of developmental (neuro) toxicity and/or grouping of chemicals.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Tuning tissue characteristics and complexity in *in vitro* models of the human intestinal mucosa

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Although a consensus is reached within the scientific community about the relevance of using *in vitro* models for the assessment of oral drug delivery via nanoparticles (NPs), the complexity and culture conditions of *in vitro* models vary immensely between laboratories. For high throughput experiments, simple monocultures of enterocytes are often used, neglecting the influence of mucus on NP permeation. On the other end of the spectrum, complex co-cultures with multiple cell types or primary material are integrated in microfluidic chips to produce highly physiological systems under flow conditions at the cost of labor-intensive, low throughput experiments. Determining the right level of complexity for answering specific scientific questions often proofs vital to uncover relevant differences of test formulations. Therefore, the definition of key physiological aspects of the mucosa and their translation into an *in vitro* model are a prerequisite for enabling reliable research. With respect to NP permeation, the tissue architecture, barrier integrity and mucus production constitute critical parameters. Although evaluations of simple *in vitro* models have been performed for an array of model drugs [1], the interaction of nanoparticles with different models of varying complexity has not been thoroughly assessed so far. Following the "as simple as possible, as complex as necessary" mindset, we cultivated attainable *in vitro* cell culture models of the human intestinal mucosa and performed a systematic evaluation with respect to critical factors for the permeation of nanoparticulate drug delivery systems. Using two NP model formulations, we assessed how the selection of cell types and culture conditions can impact transport dynamics into and through the intestinal tissue.

In vitro models of the human intestinal mucosa were cultured in Transwell® inserts with varying ratios of enterocytes (CaCo-2) and goblet cells (HT29-MTX) under static or semi-dynamic conditions. Characterization of the models was performed via histology, which allowed for the differentiation between cell types and revealed significant variations in morphology of the models depending on culture conditions. While static cultivation resulted in the formation of monolayers, dynamic conditions induced the formation of multilayered cellular structures and villi-like protrusions. *In situ* staining of mucins revealed an increase in mucus secretion depending on the ratio of goblet cells and was found to be stimulated further under semi-dynamic culture conditions. Immunohistochemistry and measurement of the transepithelial electrical resistance (TEER) gave insight into the formation of tight junction proteins and influence on the barrier integrity depending on the cell types and culture conditions. Here, *in vitro* models exhibited a decrease in TEER values with a higher proportion of goblet cells. Further, TEER values were lower in dynamically cultured tissues, mimicking more closely the reported *in vivo* TEER values of the human small intestine [2]. To study the interaction between the epithelium and NPs, two model formulations based on poly(lactic-co-glycolic acid) were used either with or without additional polyethylene glycol coating and loaded with DiI as a model substance, emulating a hydrophobic drug molecule. NPs were characterized regarding size, zeta-potential and morphology as well as cytotoxic potential, uptake into and transport across epithelial cells. Here, cytotoxicity of NPs was comparable between all models, except for goblet cell monocultures which exhibited overall lower cytotoxicity due to the protective effect of the mucus layer. Further, we observed that the three-dimensional structure of dynamic *in vitro* models decreased the uptake of NPs into the tissue, potentially due to changes in the microstructural organization of the tissue layers. While the thickness of the cell layer was found to influence permeation of both NP formulations, the mucus layer predominantly influenced the permeation of uncoated NPs.

The here presented study constitutes a systematic evaluation of *in vitro* models of the human intestinal mucosa and changes in cultivation parameters that critically influence the uptake of nanoparticulate formulations. We found that NP uptake and transport was strongly influenced by barrier integrity, mucus production and the morphology of the respective *in vitro* models. By implementing simple alterations in the rational design and cultivation of the tissue models, a higher throughput approach of more physiologically relevant models of the human intestine can be realized in most laboratories, ultimately resulting in a more efficient formulation of nanoparticulate drug delivery systems.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Cell type and developmental time-dependent effects of genotoxins on neural stem-/progenitor cell functions *in vitro*

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The development of the human nervous system is characterized by excessive proliferation of neural progenitor cells (NPCs), coupled with a high rate of apoptosis. Genotoxins can alter this balance and disturb the highly complex and time-dependent mechanisms of brain development and possibly lead to adverse effects. Additionally, error-prone or unsuccessful DNA repair can alter the differentiation capacity of NPCs. The molecular mechanisms and cellular responses, especially DNA repair capacity can thereby differ, depending on the substance. However, the available data is mostly based on rodents or cancer cells and the developmental time-dependent effects of DNA-damaging substances on human NPCs are insufficiently characterized. In this study, we analyze the effects of model genotoxins at different developmental stages of brain development by using a 3D multicellular test system with high relevance for the developing human brain.

Human NPCs allow assessment of distinct neurodevelopmental key events (KE), including NPC proliferation, radial glia migration, neuron and oligodendrocyte differentiation. Using the neurosphere assay, we analyzed the adverse effects of four genotoxins with different modes-of-action (MoA), i.e., N-Ethyl-N-Nitrosourea (ENU), Hydroxyurea (HU), 5-Azacytidine (5-Aza), and Etoposide (ETOP). The studied genotoxins altered distinct neurodevelopmental KE in the range of 0.08-200 µM and results indicate different effects of DNA-damaging substances on neurodevelopmental key events. HU, 5-Aza, and ETOP reduced the proliferation of NPCs, while all four tested genotoxins also have an impact on NPC differentiation into neurons and/or oligodendrocytes. Interestingly, the effects differed not only depending on the MoA but also depending on the treatment time point as well as the different cell types. Except for ENU, the susceptibility towards genotoxic damage declines during development in our *in vitro* model.

We showed that different model genotoxins and treatment time points lead to different human neurosphere endophenotypes. In the future, more genotoxic-related endpoints like DNA damage and DNA repair analyses will be performed to gain a deeper understanding of developmental stage and cell type-specific susceptibility of genotoxins during human brain development.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Human test methods for developmental neurotoxicity (DNT) evaluation: Set-up, scientific validation and statistical analyses

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Testing for developmental neurotoxicity (DNT) is currently performed in rats according to OECD/US-EPA guidelines. These methods are resources demanding, have unknown sensitivity and uncertainties in their interpretation. To overcome these issues and allow a large number of chemicals to be tested for DNT, a DNT *in vitro* testing battery (DNT IVB) has been assembled under the guidance of the European Food Safety Authority (EFSA) in collaboration with the Danish- and US-Environmental Protection Agency and under the umbrella of the Organisation for Economic Cooperation (OECD).

As an integral part of the DNT IVB we set up the Neurosphere Assay consisting of primary human neural progenitor cells (hNPC). This test system pictures the neurodevelopmental processes NPC proliferation and migration, neuronal and glia differentiation, neurite outgrowth as well as neuronal and oligodendroglial migration. The individual test methods representing these endpoints were scientifically validated by demonstrating specific cell morphologies, marker expressions, presence of neurodevelopmental processes, physiological signaling responses and toxicological adverse effects. 120 compounds representing different chemical classes were tested in the respective assays generating concentration-response data. Based on this data set, different biostatistical methods for data evaluation were compared with regards to compound classification and an R-based data evaluation pipeline was set up accordingly. In addition to the Neurosphere Assays, a test method for assessing human neuronal network formation and function – the human NNF assay – was established using human induced pluripotent stem cell-derived neurons and primary human astrocytes grown on microelectrode arrays. This assay was challenged with more than 30 pesticides and data was evaluated using the established biostatistical pipeline.

We present assays and testing results for a large variety of neurodevelopmental endpoints that contribute to an OECD-supported DNT IVB. More data are currently being generated to increase knowledge on the IVB's applicability domains for reducing uncertainty in its performance.

Submission declaration:
Conflicts of interest: EF, KB, AD, KK, SM and AM are shareholders of the company DNTOX that provides DNT IVB assay services.
Statement on ethics vote: There are no ethical concerns.

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An analysis of the limitations and uncertainties of *in vivo* developmental neurotoxicity testing and assessment to identify the potential for non-animal-methods 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 in the experimental animal to human real life extrapolation is uncertain.

Here, knowledge about these limitations and uncertainties is systematically summarized using an OECD format.

We also outline a hypothesis how non-animal-methods (NAM) based, 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 at least the same level of human health protection [1].

Such a conceptual comparative uncertainty discussion is not presented in the current draft *Guidance Document for the Evaluation of Data from the DNT In-Vitro Testing Battery* [2]. The purpose of the OECD draft document is just to guide the use of data from the NAM battery including their uncertainties. However, such uncertainties may be perceived as new and additional relative to those from the traditional rodent *in vivo* methods. Therefore, here it is discussed that the uncertainties indicated for NAMs in the draft OECD Guidance are in fact conceptually similar to uncertainties from the current rodent *in vivo* DNT testing and assessment: The critical effect size, in rodents and NAMs, depends on the specific test design, cannot be generalized and needs availability and consideration of positive and negative control data. Also subtle, transient effects are considered relevant, in rodents and NAMs, due to the concern for limited sensitivity and potential biological modifiers in the human world. Metabolism may be dissimilar between the test system and humans, due to rodent species and life-stage specificities or due to the isolated cellular test systems in NAMs. Kinetic and dynamic extrapolation uncertainties from point of departures, derived from rodent *in vivo* or from NAM data, to human limit values are relevant and transparent with data based probabilistic modelling approaches. The mechanistic understanding of the linkage between the measured rodent behavioral endpoints and effects on humans is limited and higher human cognitive functions cannot be tested as such, whereas also the relationships between key events tested *in vitro* and complex adverse outcomes in humans is necessarily uncertain in terms of empirical evidence and available biological knowledge. Finally, rodent *in vivo* data assessment pipelines are not standardized between *in vivo* testing facilities, but this is work in progress for the DNT *in vitro* battery.

Consequently, such NAM based testing and assessment approaches should 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 [1].

The work of Martin Paparella at the Medical University of Innsbruck is financed by the Austrian Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology, Department V/5 – Chemicals Policy and Biocides.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: not relevant

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Enabling non-invasive and chemically-selective analysis of organoid cultures using confocal Raman microscopy

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The aim of translating the complexity of human organs into cell culture systems has led to a focus on three-dimensionality in the development of *in vitro* models during the last decade. The cultivation of self-organized, miniaturized organs, called organoids, holds great potential in different fields of life sciences, from basic research to clinical applications and personalized medicine. These *in vitro* models encompass a wide array of tissues like intestinal, renal, pancreatic or lung organoids, which have been described to be implemented in disease modelling, drug development and infection research [1-3]. To allow cells to develop these complex three-dimension structures, organoids are cultured in extracellular matrix (ECM) substitutes that provide the cells with the necessary structural and biochemical cues. The commercial Matrigel® currently constitutes the go-to culture substrate, flawed by the high compositional variability, which can be attributed to its animal origin. In order to eradicate this variability and create more reliable ECM replacements, a major focal point of the research field is the development of adequate alternatives on a synthetic hydrogel basis. Although efforts are advancing, breakthroughs are impeded by the lack of sophisticated methodologies for the in-depth analysis of organoids and their interaction with the ECM. State-of-the-art analyses currently comprise non-invasive growth monitoring via light microscopy and invasive analyses like immunofluorescence staining to observe cell differentiation, flawed by limited informational output and the need of sample fixation, respectively. Although both approaches deliver valuable data for organoid analysis, they do not take into account the processes happening at the interface between the cell layer and the surrounding ECM and the resulting consequences for organoid development.

In an effort to provide a more comprehensive analysis of organoid cultures in hydrogel matrices, confocal Raman microscopy was implemented as a non-destructive analysis approach for the characterization of both organoids and hydrogel matrices. This laser-based technique allows for the chemically-selective probing and imaging of biological samples via the acquisition of Raman spectra that constitute "molecular fingerprints" of the respective specimen [4]. In a proof-of-concept study we applied confocal Raman microscopy for the analysis of murine pancreatic organoid cultures that were grown in three different ECM substrates, two synthetic formulations, varying in composition and stiffness, as well as Matrigel® as a reference and current standard.

The Raman-based analysis approach was applied to examine organoids on a single-cell level, visualizing subcellular structures without the use of labels or dyes. By interpreting the characteristic spectral features of the sample, the spatial distribution of, e.g., proteins, lipids and nucleic acids could be observed. The imaging approach was further used to visualize the interface between organoids and the surrounding hydrogel, revealing so far not accessible information about material-cell interactions. Lastly, the spatial Raman analysis approach was optimized to allow for a higher throughput evaluation by chemically probing the organoid lumen. By condensing the hyperspectral imaging to a single-point-analysis, the non-invasive method became more accessible for a fast analysis of live organoid cultures.

By investigating the interaction of cells with the surrounding ECM, we were able to assess the suitability of synthetic hydrogels to replace commercial Matrigel® and helped to identify a promising candidate based on similarities regarding organoid-matrix-interactions. The here presented proof-of-concept study provides only a glimpse into the possibilities of confocal Raman microscopy as an innovative technology to revolutionize the analysis of 3D cell culture systems in basic research as well as in a preclinical context.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

80

Development of the NPC1ab_RAR_GR assay, an *in vitro* test method to identify putative endocrine disrupting chemicals (EDCs) perturbing glucocorticoid receptor (GR) or retinoic acid receptor (RAR) signaling

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Prenatal exposure to endocrine disrupting chemicals (EDCs) is widely acknowledged to be able to disturb brain development and induce developmental neurotoxicity (DNT) [1]. However, the current regulatory risk assessment for EDCs does not cover any tests for DNT. Moreover, DNT testing relies solely on animal studies, which, aside from an insufficient testing throughput and creating ethical concerns, exhibit limited predictivity for humans due to species differences. Therefore, the refinement of the current EDC test guidelines to include new approach test methods (NAMs) identifying endocrine disruption (ED)-induced DNT is urgently required. Recent developments in the establishment of NAMs for DNT testing led to the development of a DNT *in vitro* testing battery (DNT-IVB) encouraged by the EFSA. An integral part of the battery is a multiplexed high-content assay based on human neural progenitor cells (hNPCs), the Neurosphere Assay [2]. As a member of ENDpoiNTs, a Horizon2020 research consortium focusing on the development of *in vitro* tests identifying ED-induced DNT, we adapted the Neurosphere Assay to detect disruption of retinoic acid receptor (RAR) and glucocorticoid receptor (GR) signaling based on the hormones' impact on hNPC proliferation.

Initial experiments studying hormone-dependencies of the neurodevelopmental key events covered within the Neurosphere Assay revealed that both RAR and GR activation reduce hNPC proliferation (NPC1ab). After excluding receptor interactions between RAR and GR, a test method featuring a two-tiered approach, the NPC1ab_RAR_GR, was developed. In the first tier, the test compound effect on hNPC proliferation is measured in a classical concentration-response experiment (NPC1ab) over a time course of three days. If the test compound reduces hNPC proliferation, it is labeled as a putative agonist of the RAR or GR. If no effect on hNPC proliferation is observed, the test compound is labeled as a putative RAR or GR antagonist. In the second tier, the assay is adapted to detect either agonists or antagonists of the hormone receptors by co-exposure of the test compound in presence of RAR and GR antagonists or agonists, respectively.

The NPC1ab_RAR_GR was successfully challenged with positive substances (known agonists and antagonists of both GR and RAR), as well as negative substances for receptor agonism and antagonism. Each test substance was correctly classified with the NPC1ab_RAR_GR assay. Standard operating procedures were established for this multiplexed assay, and it is now undergoing a lab-to-lab validation under the guide of the PEPPER platform.

Since hormone-regulated neurodevelopmental processes provide putative targets for EDCs and disruption adversely affects neurodevelopment in humans, the screening of putative EDCs for their potential to cause DNT is of utmost importance for several stakeholders, including the civilian population, industry, and regulatory bodies. We, therefore, aim for acceptance of the NPC1ab_RAR_GR assay on a regulatory level.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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How food science, next generation life science methods and artificial intelligence relate to European regulatory decision-making on food systems for sustainable health

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Food safety refers to handling, preparing, and storing food in a way to best reduce the risk of individuals becoming sick from foodborne illnesses. Access to sufficient amounts (SDG2 Zero-Hunger [1]) of safe and nutritious food is key to sustaining life and promoting good health and food safety is a public health priority. This becomes especially clear when looking at data from the World Health Organization (WHO), which estimates that 600 million (almost 1 in 10) fall sick each year after eating contaminated food, resulting in roughly 420,000 deaths. The EU has one of the highest food safety standards in the world [2] – largely thanks to the solid set of EU legislation in place, which ensures that food is safe for consumers. Moreover, the European Food Safety Authority EFSA carries out independent risk assessment on food and feed related issues, thus ensuring that the measures taken by the European Commission such as setting legal limits are based on sound scientific principles. In industry to market relations, food safety considerations include the practices relating to food labelling, food hygiene, food additives and pesticide residues, as well as policies on biotechnology and food and guidelines for the management of governmental import and export inspection and certification systems for foods. With the COVID-19 pandemic and the consequence of the war in Ukraine making it has been more difficult for food producers to operate with their usual workforce levels due to restrictions and supply chain issues, there has been a push into discovering the capabilities of next generation life science methods and AI.

Food Science, Next Generation Life Science Methods and Artificial Intelligence (AI) are making significant impact in the food industry. Many distinct areas of the market, from tackling food safety, security and sustainability issues, to promoting One Health [3] and sustainable process practices in food production facilities, innovating new products and personalizing product offerings through efficient and transparent regulatory processes, are benefiting from developments in food science and AI. Food safety has always been a great focus of the food industry. Failure to do so can have a drastic impact given its implications for consumer health and safety. AI and deep learning technologies have been used to establish combinations of flavors that will be popular with consumers, identify synthetic or natural modulators of body signaling pathways using next generation cell, tissue and mathematical models or accelerate, automate and harmonize regulatory chemical risk-assessment processes. Application of data-driven strategies for investigating, e.g., the gastronomic data has opened exciting avenues, giving rise to an all-new field of "computational gastronomy" [4]. This emerging transdisciplinary science applies artificial intelligence as facilitator for the identification of data-driven relations and experiments around the shape-changing nature of food for better health and nutrition.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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An *in vitro* approach to assess endocrine modes of action leading to developmental neurotoxicity

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Endocrine disrupting chemicals (EDCs) have been intensively studied regarding their harmful effects on the developing human brain. However, despite increasing evidence that early developmental EDC exposure disturbs key neurodevelopmental processes, systematic testing for developmental neurotoxicity (DNT) is not included in the risk assessment process of EDCs. Therefore, the incorporation of DNT testing strategies into the risk assessment of EDCs is urgently required. The current regulatory guidelines for the identification of chemicals causing adverse neurodevelopmental effects are solely based on animal studies. However, insufficient test throughput, species differences, and ethical concerns demand the establishment of alternative *in vitro* new approach methodologies (NAMs) and their incorporation into the regulatory framework. These test systems should be based on human cell models to ensure sufficient predictivity of the test results for the human population. According to this proposed paradigm shift in DNT testing, a DNT *in vitro* testing battery (DNT IVB) has been assembled under the umbrella of the OECD, which includes a multiplexed high-content assay based on human primary neural progenitor cells (hNPCs), the Neurosphere Assay [1,2].

Within ENDpoiNTs, an H2020 research consortium, the human Neurosphere Assay is used to identify hormone-sensitive neurodevelopmental key events and establish ED-DNT *in vitro* assays that meet the regulatory requirements for screening putative EDCs [3]. Therefore, we investigated the effects of specific hormone receptor agonists and antagonists on the neurodevelopmental processes modeled within the human Neurosphere Assay. Human neurodevelopmental key events like NPC proliferation, migration, and terminal differentiation into neurons and oligodendrocytes were significantly affected by the modulation of hormone receptor signaling. Strikingly, oligodendrogenesis was especially sensitive to endocrine modulation, being influenced by activation of the aryl hydrocarbon receptor (AhR), liver X receptor (LXR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptors (PPAR) alpha and beta/delta, progesterone receptor (PR), and vitamin D3 receptor (VDR). We incorporated both male and female hNPCs in our testing strategy, in order to study sex-specific dependencies of hormonal activity during brain development. We observed that glucocorticoid receptor modulation affected neurodevelopmental key events in male hNPCs while having no effects on female hNPCs. Moreover, we compared the effects of hormone receptor modulation in human and rat NPCs and observed that hormones influence several neurodevelopmental key events in a species-specific manner. This emphasizes again the need for human-based test systems. Since hormone-regulated neurodevelopmental processes provide putative targets for EDCs, the established ED-DNT *in vitro* assays will be used to screen libraries of known and putative EDCs and identify ED-induced DNT. Due to the dramatic consequences of DNT for human development, testing putative EDCs for their developmentally neurotoxic potential is of the highest importance for several stakeholders including regulatory bodies, industry, and the civilian population.

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Submission declaration:
Conflicts of interest: KK, AD, and EF are shareholders of the company DNTOX which provides DNT IVB assay services.
Statement on ethics vote: There is a positive ethics vote.

83

International project Train-SafeMDs: Towards the development of an *in vitro* testing strategy for local tolerance testing of medical devices used in the oral cavity

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Dental materials and dental prosthetic devices belong to the essential group of Medical Devices (MDs) broadly used widely by the general public. These MDs are in long-term contact with the oral mucosa, gingival tissues, salivary glands, and dentin and therefore safety of these products is of uppermost importance.

Most of the bio-compatibility assessments of MDs are still conducted in animals; however, thanks to the advances in tissue engineering and accelerated progress in the validation of alternative methods, the MD regulations are also implementing non-animal testing approaches. This policy has been demonstrated recently by adopting the *in vitro* cytotoxicity assays into the ISO 10993-5 and 3D reconstructed human epidermis models for intra-cutaneous testing (ISO standard 10993-23). Biocompatibility testing of MDs is usually based on the toxicity assessment of extracts into polar and non-polar solvents. That extracts are, in fact, highly diluted complex solutions/mixtures of potential irritants. Therefore, an already elsewhere validated and accepted *in vitro* test (e.g., by OECD) must be adopted to higher sensitivity levels to provide sufficient prediction and protection against unwanted side effects.

The international project Train-SafeMDs was launched in March 2020 between Austria, the Czech Republic and Slovakia and uses commercial as well as in-house prepared 3D reconstructed tissues of oral/buccal epithelia and cell cultures with the origin in the oral cavity to develop a highly sensitive testing strategy for *in vitro* biocompatibility testing of MDs.

The project predominantly aims to 1.) training of students and early-career scientists in the use of *in vitro* methods for the safety assessment of MDs, 2.) optimization of the selected *in vitro* models and methods for the MDs testing, and 3) generation of datasets with selected positive and negative reference MD materials as a part of the preparation for a broader international collaborative project.

Three practical hands-on training sessions were organized in Prague, Bratislava and Vienna. This training led to a valuable exchange of information on preparing the extracts from MDs. Furthermore, the project participants were trained in selected *in vitro* methods and generated interesting experimental data in 2D and 3D models. The team discussed and evaluated some strategies for sample preparation of complex MDs materials. The poster will present the pilot results obtained in the project.

Supported by the APVV grant DS-FR-19-0048 (Slovak Republic), MTMŠ grant 8X20026 (Czech Republic) and OEAD grant MULT 06/2020 (Austria).

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is no ethics vote applicable.

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Combining antiviral, anti-inflammatory and antibacterial properties of drugs in fighting COVID-19 disease

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Background and Objectives: Coronavirus disease 2019 (COVID-19) is a communicable disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since continuing to pose a global threat to public health worldwide, there has been a substantial effort to search for effective antiviral compounds. Based on the exaggerated inflammatory response and frequent prevalence of bacterial co-infections in patients, anti-inflammatory and antibacterial activity may confer additional benefits to a drug lead in combating COVID 19. Here, we aim at evaluating inhibitory activity against SARS-CoV-2 Main protease (Mpro), anti-inflammatory and antibacterial properties of the potential drug candidates.

Methods: Literature survey was done using PubMed, Scopus, and SciFinder® scientific search tools.

Results and Discussion: Potential antivirals target the different steps of the novel betacoronavirus replication, ranging from receptor binding, entry and fusion to replication. We systematically screened the scientific databases for compounds with anti-protease activity against Mpro, anti-inflammatory and antibacterial effects. According to the structure-activity relationship analysis, structurally diverse compounds (both natural and synthetic) were identified, pointing to a multimodal mechanism of their action. The suitable candidates will be evaluated in further *in vitro* studies regarding their toxicity and efficacy.

Conclusion: Antiviral strategy based on Mpro protease inhibition fused with anti-inflammatory and antibacterial efficiency may be a promising approach in the treatment of complicated and long-COVID-19 cases.

This work was supported by the Operational Program Integrated Infrastructure for the project: Development of products by modification of natural substances and study of their multimodal effects on COVID-19, ITMS: 313011ATT2, co-financed by the European Regional Development Fund.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Not applicable.

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IPF-on-chip: An alternative to animal models

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Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and severe lung disease characterized by progressive scarring of the gas exchange airways. Currently, only two drugs, pirfenidone and nintedanib, are available to slow the progression of the disease, but none of them allows a significant decrease of the mortality rate. Indeed, existing models are often unable to predict the effects of drugs in clinical trials. On the one hand, the bleomycin mouse model has interspecies differences and does not replicate the chronic nature of the disease as it occurs in humans [1]. On the other hand, standard lung *in vitro* models poorly reproduce the complexity of the fibrosis microenvironment. Therefore, there is an urgent need to develop relevant models to investigate key mechanisms underlying the pathogenesis of IPF and to identify potential therapeutic targets.

The lung-on-chip, new advanced lung *in vitro* models, are emerging as predictive tissue modelling tools and as a credible alternative to animal testing. These micro-engineered systems are capable of mimicking both the cellular composition and the microenvironment of the fibrotic alveoli [2-4]. We report here about the development of a IPF-on-chip model that aims at reproducing the biophysical cellular environment of fibrosis at an early and late stage. The model is based on biological membranes supported by a hexagonal gold mesh that forms an array of alveoli with *in vivo*-like dimensions [5].

The membranes are easily produced by drop casting a solution of collagen-elastin (CE) onto a thin gold mesh. A hydrogel membrane is then formed and can be used as a support for cells. The CE solution can be also dried out at room temperature within two days to form a vitrified membrane. The developed biological membranes are flexible and can be deflected in three dimensions when exposed to a negative pressure. Their mechanical properties are largely influenced by the fabrication process as well as the concentration of collagen, drying time and gelation temperature [6]. Their stiffness ranges from 1 kPa to 170 kPa, allowing the reproduction of the healthy and fibrotic tissue.

The biological membranes provide good support for cell growth and proliferation. Human primary fibroblasts and human primary epithelial cells were cultured on the vitrified membrane. It was found that the fibroblasts treated with TGF-β secreted more collagen than the untreated ones. Further studies on the effect of antifibrotic drugs on IPF cells are being conducted.

In summary, this model reproduces some key features of the lung fibrosis alveolar environment in terms of structure, extracellular matrix composition and mechanical properties. Its entire biological nature makes it a promising tool for drug discovery.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Assessment of human neuronal network formation and function using 2D and 3D hiPSC-derived cell systems

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Learning and memory are commonly affected within a wide variety of neurodevelopmental diseases and also upon chemical exposure, e.g., towards lead or methylmercury. One cellular feature of disturbed learning and memory is the inhibition of neuronal network development. This can be due to disturbed synaptogenesis as outlined in the Adverse Outcome Pathway (AOP) number 13 (AOP Wiki) on the adverse outcome (AO) "Learning and Memory". However, other disrupted neurodevelopmental processes like neuronal differentiation, neurite outgrowth or dendritic spine formation also converge on this common key event. To assess developmental neurotoxicity (DNT), an *in vitro* battery (IVB) has been established that is also supported by the Organization for Economic Collaboration and Development [1]. So far, there are no human *in vitro* assays for neuronal network formation (hNNF) in the current DNT-IVB. Hence, we developed two different *in vitro* systems for studying this endpoint.

The first test system is comprised of human-induced pluripotent stem cell (hiPSC)-derived, excitatory and inhibitory neurons as well as human astrocytes and is commercially available as the Synfire kit (Neucyte, USA). This method forms functionally active neuronal networks on microelectrode arrays (MEAs) over a time course of 35 days *in vitro* (DIV). The second test system is generated via neural induction of hiPSCs that form 3D neurospheres *in vitro*. Three-dimensional differentiation of such human-induced neural progenitor cells (hiNPCs) and subsequent plating onto MEAs form functionally active neuronal networks after 21-35 DIV. While method one assesses mainly the formation of the network itself, method two converges a variety of neurodevelopmental endpoints.

Both systems were molecularly and functionally characterized and positive and negative compounds are being tested in the systems. From these data, their applicability domains will be determined.

In vitro assays that assess the formation and function of neuronal networks will be useful tools for identifying compounds that might pose a hazard to human learning and memory. Studying tool compounds in different assays will help define applicability domains for different assays that will improve their use for application.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Refinement of an *in vitro* testing battery for developmental neurotoxicity assessment by integration of radial glia- and astrocyte-specific endpoints

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Considering the resource-intensity and ethical concerns of the current *in vivo* test guidelines for developmental neurotoxicity (DNT) testing, new tools and methods are required to strive towards a more efficient and sustainable risk assessment paradigm. The use of human-based *in vitro* new approach methodologies (NAMs) complementing or in the future replacing animal studies can advance toxicity testing in terms of its ethical, economic and also scientific limitations. Recently, a DNT *in vitro* testing battery (DNT-IVB) has been assembled in cooperation with the EFSA and the Danish and U.S. EPA under the umbrella of the OECD and was challenged with 119 chemicals [1]. Despite its broad coverage of neurodevelopmental key events, a gap analysis indicated that the integration of test methods based on radial glia cells (RG) and astrocytes (AC) could improve battery performance [1]. Therefore, as part of a Cefic-LRI-funded research consortium, we are establishing novel human-based test methods for measuring RG morphology and proliferation as well as for AC differentiation, maturation, and function.

The additional test methods will be based on the human Neurosphere Assay, which is a high-content assay that is already part of the current DNT-IVB. This assay is based on primary human fetal neural progenitor cells (hNPC) cultivated as proliferative neurospheres that have the potential to differentiate into brain effector cells including radial glia cells, neurons, oligodendrocytes, and astrocytes. Upon cell plating on an extracellular matrix, hNPCs radially migrate out of the sphere core, thereby forming a circular migration area [2].

Within the first 24-48 h of hNPC differentiation, the culture predominantly consisted of nestin-expressing cells displaying typical RG-like morphology. To enhance proliferation in the migration are, hNPCs are cultured in presence of a defined EGF/FGF2 ratio allowing for the simultaneous assessment of RG migration, morphology and proliferation. Therefore, immunocytochemical staining for the proliferation marker Ki67 in combination with Phalloidin is applied to detect proliferating cells and to visualize actin networks. The EGF receptor inhibitor PD153035 was used to antagonize the growth-factor induced effect on proliferation. Treatment with the microtubule depolymerizing reagent Nocodazole resulted in alterations of RG morphology and concomitant cell migration. Since these chemicals were shown to reliably alter the respective RG-related endpoints they will be used as positive controls for this test system.

AC differentiation is promoted by a combination of BMP2 or 4 and CNTF and evaluated after 5 days of differentiation by immunocytochemical staining for AC-specific markers (e.g., AQP4, GFAP) and qPCR analyses. After 5 days of hNPC differentiation, an almost pure culture of cells positive for the AC-specific marker AQP4 (> 95%) was obtained, which displayed the typical star-like cell morphology. Gene expression analyses confirmed that the differentiated cells express AC-specific markers (S100b, AQP4, GFAP). The data indicates sufficient maturity of hNPC-derived AC. Functional characteristics will be assessed prospectively by measuring the glutamate uptake capacity and reactivity of the generated AC culture.

Following the establishment of the new RG- and AC-based DNT test methods, they will be used to detect chemical interference with human RG and AC differentiation after exposure to a potential DNT compound. The Selection of chemicals will be based on known DNT positive and negative compounds as well as false-negative (FN) substances identified within the EFSA-coordinated DNT-IVB performance testing. We hypothesize that the FN rate of the classical DNT-IVB can be reduced by introducing RG- and AC-specific endpoints into the current DNT-IVB. By enhancing the battery performance, we will increase the predictivity of testing results and the confidence in implementing the DNT-IVB as the new gold-standard of *in vitro* DNT testing.

References

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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"DVM: Training the animal doctor" – A documentary film series

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The curriculum for veterinary education and training at every institution continually undergoes revision to ensure quality and relevance. At the scale of the student practical class and professional development training course, the tools and approaches employed will change as technology provides new opportunities and as ethical and animal welfare concerns are given greater prominence. A documentary film series has been produced by InterNICHE to explore humane innovations that can enhance acquisition of knowledge, skills and attitudes at university and beyond. The main target demographic includes university course leaders, heads of department, administrators and students. This presentation will feature the documentary summary.

Based on site visits to universities across the world, the documentary showcases selected university departments and faculties that are at the forefront of progressive change in veterinary education and training. It includes interviews with educators, students and producers who share their experience of the development and implementation of humane methods. Demonstrations and student practical classes illustrate their use for teaching and learning, from comparative anatomy to gaining competency within abdominal surgery. The range of tools and approaches featured include virtual laboratories and virtual reality software for anatomy, physiology and pharmacology; client donation programs to provide ethically sourced cadavers; advanced synthetic models and mannekins for hands-on clinical skills and surgery training; scenario-based simulated clinics for internal medicine; and clinical learning opportunities with animal patients.

The documentary provides a comprehensive overview and multiple case studies of humane tools and approaches designed to improve education and training through the better meeting of teaching objectives. It explores the broader pedagogical, scientific, ethical, economic and environmental advantages of these innovations. It also demonstrates the feasibility of replacement of any harmful animal use that might remain within the field, such as dissection of purpose-killed animals, animal experimentation, and other instrumental animal use. The impact of this curricular transformation is to the benefit of the students, the educators, the animals, the veterinary profession, and society itself.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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High sensitivity of stem cell-derived motor neurons to pharmaceutically relevant botulinum neurotoxins A1, B1, E1 and F1

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Around 400,000 mice are used annually in Europe for potency testing of pharmaceutical preparations of Botulinum neurotoxins (BoNTs) in the mouse bioassay, although some product-specific proprietary *in vitro* methods are already in use. BoNTs cleave proteins of the soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) complex, involved in exocytosis, and therefore inhibit the release of neurotransmitter containing neuronal vesicles at the neuromuscular endplate, inducing a flaccid muscle paralysis. Currently approved alternatives for the mouse bioassay are product-specific, as they mostly identify serotype-specific protein cleavage. Since our aim is to develop a cell-based and serotype-independent method for BoNT potency assessment for replacement of the mouse bioassay, this study focused on sensitivity assessment of induced pluripotent stem cell (iPSCs)-derived motor neurons (MNs) to pharmaceutically relevant BoNT serotypes A1, B1, E1 and F1.

MNs were differentiated from iPSCs according to three different protocols and their sensitivity to BoNT/A1, /B1, /E1 and /F1 was assessed by quantification of substrate cleavage via Western blot with different antibodies. Antibodies for detection of the full substrate SNAP25, cleaved by BoNT/A1 and /E1 and for detection of the full substrate VAMP2, cleaved by BoNT/B1 and /F1 were used as well as the neoepitope antibody BoNT/F/440 to detect VAMP2 site-specific cleavage by BoNT/F1 [1]. EC_{50} activity values were compared to Mouse LD_{50} values from the mouse bioassay.

MNs are highly sensitive to the currently pharmaceutically used BoNT serotypes A1 and B1, with the most sensitive protocol for MN differentiation (Maury) showing an EC_{50} of 0.034 pM translating into 0.05 Mouse LD_{50}/mL to BoNT/A1 being 20 times more sensitive than mice, in the Western blot setup assessing substrate cleavage by detection of the full substrate. An EC_{50} value of 0.69 pM translating into 4.12 Mouse LD_{50}/mL to BoNT/B1, four times less sensitive than mice could be measured respectively [2,3]. This most sensitive differentiation protocol was chosen for sensitivity assessment of MNs to BoNT/E1 and /F1 and showed an EC_{50} of 0.77 pM translating into 4.54 Mouse LD_{50}/mL for BoNT/E1 and an EC_{50} of 0.80 pM translating into 2.46 Mouse LD_{50}/mL for BoNT/F1, assessing substrate cleavage also by detection of the full substrates SNAP25 and VAMP2, respectively. For BoNT/F1 a neoeptitope antibody for detection of the specific cleavage site at VAMP2 was used additionally to compare results between the two detection methods. EC_{50} values were 4.36 pM for the full substrate antibody, and 1.7 pM for the neoepitope antibody differing significantly, analyzed by non-convergent 77.6% confidence intervals after non-linear regression of the data.

Demonstrating high and human-relevant sensitivity, iPSC-derived human MNs seem to be well suited for serotype independent BoNT potency testing. For BoNT/A1 we saw 20 times increased sensitivity compared to the mouse bioassay, while for the other serotypes a decrease of 2 to 5 times was demonstrated. Especially when it comes to BoNT/B1 sensitivity, it is known that humans are 30 to 100 times less sensitive than mice, due to a receptor mutation in synaptotagmin 2 involved in BoNT/B receptor-mediated endocytosis. Therefore, this slightly reduced sensitivity could nevertheless be relevant for BoNT potency assessment for human pharmaceutical use. Further, this Western blot-based sensitivity assessment still shows high variability and serves only for the choice of the most sensitive protocol for the further development of a cell-based activity assay, expected to be more sensitive.

Funded by the German Federal Ministry of Education and Research (FKZ 031L0132B).

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote required.

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Development of an innovative cartridge bioreactor for parallelized cultivation and stimulation of complex tissue models

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Musculoskeletal disorders are among the most common health problems, and the number of affected patients is increasing due to the aging population. Adequate patient care is only possible if appropriate new therapeutic strategies pass through the preclinical and clinical phases and eventually reach the market. Especially in the preclinical phase, the use of small- and large-animal models is still relied upon, as sufficiently suitable *in vitro* models are not yet available.

To address this gap, we have successfully developed a preclinical 3D *in vitro* model that simulates the initial phase of fracture healing by culturing fracture hematoma models with bone models composed entirely of self-assembled mesenchymal stromal cells for up to 48 hours in a still static environment [1,2]. Although we have already demonstrated similar cell composition, gene expression and response to the osteogenic inducer deferoxamine compared to *ex vivo* and *in vivo* data, our preclinical *3D in vitro* model still lacks features such as mechanical loading and perfusion that are known to influence fracture healing.

Therefore, we are currently developing a modular system based on cartridges integrated in a bioreactor enabling us to cultivate 3D *in vitro* model simulating the initial phase of fracture healing *in vitro* under well-controlled conditions (temperature, pH, humidity, biomechanical stress, perfusion rate). The resulting innovative cartridge bioreactor will allow us to (i) closely mimic the *in vivo* situation of fracture healing and its disorders but also other bone related diseases, (ii) analyze biomaterials and other therapeutic strategies as well as (iii) the impact of extreme gravitational forces (e.g., zero gravity) with regard to mechanical stimulus (iv). The cartridge bioreactor will additionally enable us to extend cultivation time in order to study the transition towards the anti-inflammatory phase with respect to perfusion rate.

Ultimately, we will obtain a sophisticated multidimensional cartridge-based analytical bioreactor suitable for integrating models of bone and other components of the musculoskeletal system, including muscles, tendons and joints.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

93

Risk assessment of microplastic by *in vitro* genotoxicity assays

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The uptake of microplastic (MP) can threaten human health. Additionally, to the common belief of MP mainly being ingested by the consumption of seafood, it was recently proven that MP pollution in urban areas is a major problem. The daily contact with plastic additives as bisphenol A, phthalates, and styrene oxide that easily migrate for instance from food packaging into food or the inhalation of MP particles created by rubber tire wear have raised major concerns. However, detailed knowledge about adverse effects of MP upon uptake by humans is still limited.

In this study we aim to establish standardized *in vitro* genotoxicity assays in mammalian cell lines to assess possible genotoxic effects of MP particles and leachates of plastics. First, the comet assay (OECD 489) was established to analyze DNA damage in Balb/c-3T3 mouse fibroblasts. Furthermore, the *in vitro* mammalian cell gene mutation test (TK-mouse lymphoma assay, OECD 476) was established in L5178Y $TK^{+/-}$ mouse lymphoma cells. Appropriate positive and negative controls at the presence and absence of metabolic activation (S9 fraction) were included.

Having established these *in vitro* test systems, we will in future investigate the putative genotoxic potential of MP and other environmental risk factors.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: An ethics vote is not required.

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Comparative analysis of chemical composition and permeability properties of artificial and excised skins – Reduction and replacement

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During the last decade an increasing interest can be observed in the field of use of *in vitro* skin substituents for reduction of the number of the experimental animals sacrificed for pharmaceutical and dermatological studies. The most relevant models of course are the human skins, but the availability of these tissues is very limited. The big advantage of the *in vitro* reconstructed skin equivalents is that they can be generated from human cells, and therefore they are relevant, have a good predictive potency and low variability. The use of full thickness three dimensional (3D) skin models and human reconstructed epidermis (HRE) is widespread in irritation, penetration and toxicological studies [1-3]. However, the structural mapping of these tissues and in parallel their functionality has not been analyzed yet.

Studying skin composition and interaction with topical substances is important in dermatology, drug safety and cosmetoscience. Several techniques are utilized and are under development for these purposes. Skin models are in use with different complexity from 3D bioprinted skins through human reconstructed skin substituents to excised tissues. These models are successfully applied in diffusion cells and skin-on-a-chip devices. In the current study two model drugs (caffeine and quinidine) were investigated as a cream formulation in microfluidic skin-on-a-chip device and by confocal RAMAN spectroscopy. Excised skins and skin substituents were compared in regard of their chemical composition, barrier function and permeability. First the measuring system was optimized and then the properties of the skin models were characterized. Caffeine, as a hydrophilic drug easily penetrates through the skin, the more lipophilic quinidine reached a much lower concentration in the perfusion fluid. The accumulation of the drugs in the upper layers of the epidermis was similar showing that quinidine can well-penetrate to the lipophilic matrix of stratum corneum but does not cross the full thickness skin barrier as easily. In the penetration of caffeine, the transappendageal route has a crucial role, this can explain the two order of magnitude difference in absorption compared to quinidine. Furthermore, quinidine interacts with several transporters in the skin (especially P-gp) which influences its penetration profile.

The findings of current research confirm that HRE can be a good substitute of human or animal tissues in diffusion studies also in skin-on-a-chip, although there are some differences in the composition of the structural elements between HRE and skins. In addition, the pathological human obese skin tissues can be less relevant models in pharmaceutical studies than HRE for prediction of drug penetration and accumulation in physiological or dermatological conditions (like psoriasis, atopic dermatitis and inflamed or dry scaly skin). For testing drug absorption in skin disorders appropriate preclinical models are available [4,5] but these should also be substituted with *in vitro* cell culture model systems in the future. To analyze the skin composition in the various dermatological conditions further studies are needed.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Characterization of human brain endothelial cells for the development of *in vitro* blood-brain barrier models

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The blood-brain barrier (BBB) is a highly selective semipermeable barrier, which controls the influx and efflux of substances, and maintains the metabolic activity and neuronal function in the brain. *In vitro* BBB models play an important role in investigating the cellular and molecular mechanisms in the context of drug discovery or the assessment of bio-reactive compounds to overcome the BBB. Since brain endothelial cells are the key component for the formation of the barrier, they represent the first cellular target when evaluating drug delivery into the brain parenchyma [1]. Here, we compare the human immortalized brain endothelial cells, hCMEC/D3, with the human primary brain microvascular endothelial cells (hBMVEC) for their barrier characteristics as well as the expression of BBB-relevant markers.

We demonstrate that both cell types express important BBB markers such as the tight junction (TJs) proteins, ZO-1 and Claudin-5, and the influx and efflux transporters, transferrin receptor, Glut-1 and P-glycoprotein with immunofluorescence or qRT-PCR. In addition, primary cells expressed 4-fold higher level of Claudin-5 compared to immortalized cells as assessed by flow cytometry analysis. The tightness of the barrier was monitored by measuring the transendothelial electrical resistance (TEER) under static conditions in the transwell system over several days of cell cultivation, hCMEC/D3 cells produce a sub-physiologic TEER with a maximum of 15 Ω*cm^2 after 7 days of cultivation. However, the primary cells reach their maximal resistance of 300 Ω*cm^2 when cultivated for 3 days indicating higher barrier integrity.

Overall, we could demonstrate that primary cells have better barrier properties by expressing higher levels of TJs, as well as reaching higher TEER. Therefore, they can be used to produce a BBB model with more physiological features. As a drawback, primary cells in culture lose their BBB phenotype over time. Other disadvantages are access to sufficient cells and donor-to-donor variations. In contrast, immortalized cells, such as hCMEC/D3, are stable and easy for cell cultivation but lack BBB-specific barrier tightness. However, since the hCMEC/D3 show high reproducibility and a stable BBB phenotype, they represent a good model for transcytosis and transport mechanism studies. Nevertheless, endothelial function is highly dependent on intercellular interactions and flow-related shear stress, which needs to be considered for future assessments.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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3D bioprinted cancer model as an efficiently applicable drug testing platform

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As cancer is a major cause of death worldwide, developing efficient treatment options for many tumor types and especially for metastatic cancer has been an urgent demand nowadays. The cancer microenvironment is known for being complex, heterogeneous, and therefore difficult to study in two-dimensional (2D) models. To this end, animal models were developed, but the application of currently available animal models is still hampered by the fact that human cancer cells are embedded in an animal-derived environment. This xenogenic microenvironment often has limited relevance to human pathophysiology, resulting in very low success rate for the translation of insights from animal models to clinical trials, and a high failure rate of drug candidates in oncology.

In the last few years, there have been many advancements in the field of 3D bioprinting. This technology allows the fabrication of complex 3D functional living tissues and even artificial organs. It is not only showing the broad prospects in personalized treatment, but also regarded as a very important alternative for conventional research methods, such as 2D cell culture and animal experiments. For cancer research, these bioprinted constructs have been demonstrated to have the potential to recapitulate human cancer tissues or microenvironment. One of the main purposes for 3D bioprinting cancer models is to exploit potential cancer therapies, especially in screening trials of anti-cancer drugs.

Our lab successfully developed different kinds of 3D models by 3D bioprinting for various studies. Here, 3D bioprinted cancer models are introduced as an efficiently applicable drug testing platform. Neuroblastoma is the most common extracranial solid malignancy of childhood. Major obstacles include managing chemotherapy-resistant relapses and resistance to induction therapy, leading to early death in very-high-risk patients. We therefore developed a 3D model of neuroblastoma renal cancer. It is composed of neuroblastoma (IMR-32 cells) with amplified genes of the *myelocytomatosis viral related oncogene MYCN* and the *anaplastic lymphoma kinase (ALK)* in a renal environment of exclusively human origin, made of human embryonic kidney 293 (HEK293) cells and primary human kidney fibroblasts. The model was produced with two pneumatic extrusion printheads using a commercially available bioprinter. Two drugs were exemplarily tested in this model: While the *histone deacetylase (HDAC)* inhibitor panobinostat selectively killed the cancer cells by apoptosis induction but did not affect renal cells in the therapeutically effective concentration range, the peptidyl nucleoside antibiotic blasticidin induced cell death in both cell types. Importantly, differences in sensitivity between 2D and 3D cultures were cell-type specific, making the therapeutic window broader in the bioprinted model and demonstrating the value of studying anticancer drugs in human 3D models. Altogether, this cancer model allows testing cytotoxicity and tumor selectivity of new anticancer drugs, and the open scaffold design enables the free exchange of tumor and microenvironment by any cell type [1].

Recently, we further developed a more complex cancer model with perfusable blood vessels. This cancer model recapitulates hepatic metastasis of neuroblastoma, where neuroblastoma is surrounded by hepatic tissue. To fabricate the perfusable cancer model, the liver cells (HepaRG or hepatocytes) and neuroblastoma (NB-1 cells) were encapsulated into the bioink and printed using a mechanical extrusion-based printhead and an inkjet printhead, respectively. During the printing process, the vessel-shaped channels were formed directly, and then coated with vascular cells. A customized perfusion setup was designed to enable the medium flowing through the perfusable cancer model, so that the perfused medium can supply the nutrients and oxygen for the whole construct. Moreover, the anti-cancer drug Ceritinib, an ALK inhibitor, was given into the perfused medium to simulate the drug dosing scenario *in vivo*. Consequently, the treated cancer model was characterized by various methods to detect the response of the cells and evaluate the therapeutic effect of the drug. Therefore, the perfusable cancer model presented here is capable of studying the function and unforeseeable side effects of potential therapeutics. We believe the combination of the human derived environment and bioengineered vascular network will facilitate the studies of new therapeutic screening, individual medicine treatments, and the investigation in progression of the cancer metastases.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Not applicable.

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Advanced *in vitro* models to study molecular mechanisms of Alzheimer's disease

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the accumulation of extracellular amyloid beta (Abeta) plaques, formation of intracellular neurofibrillary tangles and neuro-inflammation leading to loss of neurons and synaptic connections [1]. Despite the progress in research during recent years, the underlying molecular mechanisms of the widely accepted "Abeta-hypothesis" have not been fully elucidated. Recent findings indicate that toxic oligomers of Abeta may cause propagation of the pathology to healthy neurons, although the underlying mechanisms are incompletely understood [2]. Different mechanisms of propagation from diseased to healthy neurons are described: via exosomes, synapses, or tunneling nanotubes [3]. Animal models have proven poor human relevance and are sources of ethical concern. As an alternative to animal models, we aim to establish and apply an *in vitro* cellular model to distinguish between the above-mentioned mechanisms of propagation of AD. The goal of the work is to evaluate if the diseased phenotype can be propagated *in vitro* and whether factors released into the medium suffice or if cell-cell contact is necessary.

We generated familial AD (FAD) cell line with mutations in human amyloid precursor protein (APP) and human presenilin 1 (PSEN1) using neural progenitor parental ReN (ReN-FAD-GFP) cells based on [1]. ReN-FAD-GFP were considered diseased cells, ReN-GFP were used as non-diseased control line, and parental ReN cells represented the healthy population. In our previous studies, ReN-FAD-GFP neurons grown in thin layers of Matrigel exhibited a higher number of Abeta deposits and hyperphosphorylated Tau after 6 weeks and 9 weeks of differentiation, respectively. Here, we cultured ReN-FAD-GFP or ReN-GFP together with ReN parental cells in a thin layer of Matrigel. We used different configurations that allowed medium exchange between the cell populations with or without direct cell-cell contact.

The data show that 8-week differentiated ReN parental cells, which shared medium with ReN-FAD-GFP cells (non-contacting culture) showed signs of cell damage, reduced neuronal networks and increased LDH release compared to ReN parental cells sharing medium with ReN-GFP control cells. Moreover, 12-week differentiated ReN parental cells treated with ReN-FAD-GFP conditioned medium showed higher cell damage and less neuronal connections compared to control parental ReN cells.

In conclusion, our data indicate that released factors such as exosomes and/or free soluble mediators are sufficient to promote the propagation of the AD phenotype between diseased and healthy neurons. This *in vitro* model represents a useful tool to identify means of intercellular communication. In this context, such a cellular model could help elucidate the pathological mechanism of AD and accelerate drug discovery by identifying new targets for the treatment of AD patients. Future steps will include the analysis of exosomes, and the release of soluble factors (e.g., release of Abeta release by ELISA).

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote

98

An *in-vivo* engineered chip as a smart intravital multiphoton imaging window for new validation protocols of biomaterials

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The current protocols for biocompatibility assessment of biomaterials (ISO 10993-6 [1]), based on histopathology, require the sacrifice of a huge number of laboratory animals for *ex-vivo* analysis, with an unsustainable ethical burden and remarkable costs. Intravital microscopy techniques can be used to study implantation outcomes in real time with some restriction on the spatial resolution due to the limited light penetration in deep tissues that can be overcome partially by means of implantable imaging windows. However, they are cumbersome to implant, having limited capabilities in quantification of the reaction in longitudinal studies.

We present here the outline and first tests of a novel chip, whose aim is to enable longitudinal studies of the reaction to the biomaterial implant. The chip, object of the EU FET-OPEN project IN2SIGHT, is composed of a regular reference microstructure and a set of microlenses, both fabricated via two-photon polymerization in the SZ2080 resist [2]. The photoresist composition and architecture of the microstructure (geometrical design and planar raster spacing) largely determine the mechanical and spectroscopic features of the microstructures [3]. The microlenses will act as the final component of a microscope objective, allowing to enter with quasi-collimated light in the tissue, thereby minimizing the spherical aberrations due to refraction index mismatch.

The chip is designed to be implanted in mice and with the aim to observe tissue reactions under the non-linear excitation microscope for up to 2 weeks. The first *in vivo* validation of the In-2Sight chip, presented here, was performed in living chicken embryos by fluorescence microscopy, 3 and 4 days after its implant. The quantification of cell infiltration inside the chip demonstrates its potential as novel scaffold for tissue regeneration to realistically model the interface with a biomaterial implanted *in vivo*.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 964481

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Effects of plastic on the reproductive behavior of the freshwater snail *Biomphalaria glabrata*: An invertebrate *in vivo* study

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Plastic has been an increasingly important omnipresent material with versatile properties and diverse applications since the 1950s. However, its long life and decomposition into microplastics, and its various additives cause significant environmental pollution with growing scientific and public health concerns. Risk assessment of microplastic pollution is highly demanded, however often includes animal testing.

In this study, we investigated the putative effects of conventional (high density polyethylene) and biodegradable plastic bags (based on corn starch) on the aquatic system, using the freshwater snail *B. glabrata*. In comparison to vertebrates, invertebrate study systems are less regulated and do not need approval by an ethical commission. Nevertheless, efforts are done to keep invertebrate research ethical. Therefore, all experiments were planned carefully, and highest attention was paid to avoid any suffering. In addition, a non-invasive assay was chosen to investigate the reproduction behavior of adult snails.

Snails were exposed to pieces of conventional and biodegradable plastic bags, as well as to leachates of the two plastic bags, which potentially contain harmful substances. The reproduction behavior was monitored by counting the number of laid egg clutches. Our results demonstrate that the reproductive behavior of *B. glabrata* was positively influenced by plastic, whereby the increased clutch production was possibly not affected by leached chemicals but rather by other stressors such as the surface area of the plastic or the number of plastic pieces. Although further experiments are needed to gain more information on the impact of plastic on aquatic organisms, our study demonstrates that *B. glabrata* is an appropriate *in vivo* invertebrate test system for ecotoxicological risk assessment.

Financial support from the City of Vienna project “Microplastic and Nanoparticles as Environmental Risk Factors” (29-13) is gratefully acknowledged.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The relevance of human primary material in animal free research – Orthopedic trauma as an example

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Animal free research is the aspired future of biomedical research. In order to abandon animal research, it is necessary to rethink infrastructure and resources regarding patient material and clinical trials. Working closely with clinicians is still a matter of personal engagement, ease of access and the random advantage of funding to enable patient related research.

The infrastructure of clinical trials is a promising path to intertwine research with medical interventions. Patient heterogeneity is much needed in these trials and should be considered in basic research as well. Clinically relevant research should include the cohort of people, which are affected by the disease or pathology [1]. Inbred, sterile humanized mice are not representative of the comorbidities a patient is suffering, which might be quite common for his or her* age or gender group. Standardization must be reached through other parameters. In orthopedic surgery, which can include fracture management besides joint preservation and replacement surgeries, the parameters can include fracture location, trauma severity, concomitant injuries, time point and healing period, medication or common comorbidities such as osteoporosis and rheumatoid arthritis. Since some of the patient cohorts usually share similarities in terms of accumulation of existing diseases and distribution of age, weight and gender as a cofactor, it is possible to draw some conclusions about their biological comparability. This could mean that their similarity outweighs their diversity. If one intervention is suspected to have an effect, it is important to understand whether the effect is influenced by commonalities in the group or a new observation. If the effect remains, we can draw conclusion from underlying mechanisms maintained despite our uniqueness. As an example: fractured human bone marrow always shows signs of activation after trauma. This can be measured with activation markers on human T and B cells and monocytes present in human bone marrow biopsies from femoral neck fracture patients. It is of essence to minimize technical mistakes, such as varying time points of sample retrieval and processing or varying storage conditions. In arthroplasties, wear and corrosion products influence the quality and the stability of the implant. Bone marrow cells including resident immune and bone cells are impaired by high metal levels, despite the wide variety of implant designs, types and alloys used. Documentation of relevant details must be ensured by a well-established line of communication between clinician and researcher and according to data protection laws and ethic approvals [2].

After assessing patient material and analyzing samples to determine findings of interest, the findings must be validated in *in vitro*. Our bone and bone marrow chip has served as a robust model to mimic the distribution of metal debris in bone tissue and marrow. Primary patient cells were isolated seeded onto 3D bone scaffolds and despite the varying levels of osteogenic potential and immune cell composition, metal debris seems to destroy bone marrow matrix and impairs cellular functions [3]. The same model is now used to determine the findings from fracture patient bone marrow. With patient cells, we try to reconstruct the timeline of bone marrow activation due to trauma. To ensure this kind of research, we established a pipeline and a workflow from bedside to bench. It is crucial to arrange sample retrieval and optimal handling of heterogeneous donor material as well as direct and clear communication with the health care professionals and a cooperating clinician on site. Usually, this process is up to the personal determination of all workers involved, therefore a structural guideline and implementation is needed. A standardized system should be developed and integrated into all institutes and groups working in biomedical fields and especially translational medicine. It should include a monitor or a person fulfilling the role equivalent to an animal protection commissioner. This person would support scientists to utilize material generously donated by patients and help them with structural hurdles, paperwork, ethics, guidelines, legal issues and data protection [4]. Only then, it is possible to fully promote animal free but patient-specific research to finally minimize animal experimentation and promote 3R or replacement methods.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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"Improve your Skills" – The innovative 3R-Skills lab of the 3R-Centre Giessen

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The use of animals for educational purposes is increasingly scrutinized in public perception as well as by responsible authorities when applying for teaching permits. Throughout Europe, there are clear efforts to reduce the number of animals used in experiments, also in the educational context. The ICAR3R- 3R Centre of Giessen now established a 3R-Skills Lab "Improve your Skills" for future researchers but also for those already actively involved in animal research. The main goal of "Improve your skills" is raise awareness for the 3Rs and to promote the willingness to critically rethink the use of animal models.

Knowledge and skills are taught through work at stations. This enhanced the usage of digital media in didactic concepts towards future-proof learning. At the stations teaching videos, simulators and real equipment from laboratory animal husbandry offer an authentic inside in laboratory animal science and a contemporary teaching to support the learning of theoretical content and practical skills. This results in a balanced amount of haptic, visual and acoustic components to appeal to and inspire every type of learner.

All the 3Rs are addressed. By this, replacement-methods, measures to reduce the numbers of animals used for scientific purposes as well as refinement methods are outlined, whereby the individual animal and its ability to suffer is always the major focus.

Today, the Skills Lab makes an enormous contribution to the reduction of laboratory animals and the refinement of methods used in experiments. In the future, it raises questions about the general use of animals for scientific purposes in order to contribute to the long-term goal of elimination of animal experiments.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The electro-mitochondrial coupling of a microphysiological human heart

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Cardiovascular disease is one of the leading causes of death worldwide. Efforts to study cardiac function are often limited by physiological differences between human and small animal models, leading to a surprising number of adverse events in drug development. Recently, we have developed a sensors-embedded, hiPSC-derived model of vascularized multi-chambered, synchronized, self-paced cardiac organoid. Our sensor-embedded platform permits the simultaneous real-time measurements of oxygen uptake, extracellular field-potential, and cardiac contraction with >10-Hz resolution. We have shown 1-Hz cardiac respiratory cycles whose frequency is coupled to the electrical rather than the previously theorized mechanical activity of the tissue. Microscopic analysis reveals that calcium oscillations in human cardiomyocytes drive mitochondrial respiration in preparation for mechanical contraction. Importantly, pharmaceutical inhibition of this electro-mitochondrial coupling leads to arrhythmogenic behavior. This work illuminates new metabolic dynamics of cardiac rhythms, underscoring the utility of microphysiological systems for advancing our understanding of cardiac physiology. This work sheds light on an important behavior of cardiomyocytes, suggesting that mitochondrial dysfunction may drive arrhythmogenic events, underscoring the utility of hiPSC-based cardiac microphysiological systems for advancing our understanding of cardiac physiology. We will combine the data to provide a real-time assessment of the organoid's function and health, allowing us to investigate response for prediction of drug- induced cardiotoxicity, perfusion injuries and modeling circadian rhythm. Our microfluidic platform will reveal the dynamics and strategies of cellular adaptation to cardiac damage, a unique advantage of organ-on-chip technology.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Human induced pluripotent stem cell-derived alveolar epithelial cells: A comparative gene and protein expression analysis between 2D- and 3D-differentiation

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Human induced pluripotent stem cell (hiPSC)-derived lung cells are a promising cell source for reliable *in vitro* models in drug development or toxicity testing. Since simple cell lines do not exactly represent the lung physiology and primary cells are rare to get and limited to cultivate, alternative physiological cell sources are highly demanded [1].

To date, hiPSC can be differentiated into several lung cell types and organoids with varying outcomes [2-5]. The success of differentiating hiPSCs into lung cell types depends on how complex embryonic developmental pathways are mimicked *in vitro* and how a cellular environment including the interaction with extracellular matrices or the formation of tissue-specific milieus can be provided. Properties, which can rather be achieved by three-dimensional (3D) than two-dimensional (2D) culture conditions [6]. However, from existing protocols it remains unclear, if differentiation into alveolar epithelial cells (AECs) from hiPSC is limited to 3D conditions or whether 2D approaches are also suitable for the generation of AECs. Additionally, it is not known so far, if 2D- and 3D-differentiated AECs from hiPSC differ in their gene and protein expression pattern regarding specific lung markers during the embryonal lung development simulated under *in vitro* conditions.

A reproducible protocol suitable for both 2D- and 3D-differentiation of the predominate cells of the alveoli, type1 and type 2 AECs (AEC1 and AEC2), has been established to perform a comparative analysis in five different hiPS cell lines (from healthy and Cystic Fibrosis donors) within five developmental stages of the lung. A dynamic suspension bioreactor was used to differentiate AECs under shear stress and homogenous nutrient supply, whereas 2D-differentiated cells were generated under standard static conditions.

Significant differences between 2D- and 3D-conditions, especially on gene expression level during the first embryonal stages, but also in the final AEC stages could be determined. Moreover, strong differences in the gene expression of lung surfactant proteins could be revealed in comparison to adult human lung.

Finally, the gained knowledge helped to identify the 3D-bioreactor based differentiation approach as a promising method to generate high amounts of AECs for alveolar *in vitro* models for drug development and toxicity testing, but also revealed that even simple 2D-differentiation approaches are suitable to generate these cell types from healthy and disease hiPS cell lines.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Not applicable.

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Direct replacement of secondary antibodies by Affimers

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Antibodies are widely known as the "gold standard" of affinity reagents in the fields of clinical diagnostics, therapeutics and research. Monoclonal and polyclonal antibodies are used and whilst mAbs can be produced *in vitro*, pAbs still rely on animal immunization and sacrifice. This requires the slaughter of thousands of animals each year and furthermore leads to high levels of variation between batches of antibodies produced [1,2]. Secondary antibody reagents represent a high proportion of pAbs produced in the research sector. Secondary antibodies are used to detect the constant region of a range of primary antibody targets within an antibody class, which in turn detect a specific protein. This system is used with the aim of amplifying the signal provided by the primary antibody. The literature surrounding synthetic binding protein scaffolds has recently been expanding and gaining more notice, particularly as possible alternatives to antibodies [3,4]. Nevertheless, limited exploration has been carried out in research settings, something that this project hopes to change. The scaffold family of Affimers have shown a wide range of potential uses and versatility [5]. They have been used in 100s of phage display screens and are easily accessible, having been developed in Leeds. This project is investigating the use of Affimers as replacements for secondary antibodies in research with a focus on improving production methods and potential benefits to the data produced. Phage display against the constant regions of mouse and rabbit IgGs has been carried out to isolate binders to be implemented in place of secondary antibodies in techniques such as Western Blotting, ELISAs and immunochemistry. So far over 20 Affimers have been isolated against mouse antibody constant regions and binding has been confirmed using phage ELISAs. The sequences of these Affimers are diverse suggesting that there could be a range of binding sights in use on the target antibody. The Affimers isolated so far seem to be very selective and specific to their antibody subclasses, a potential benefit leading to lower background activity in downstream uses. These results are promising for ultimate aim of this project, the creation of an Affimer "polyclonal secondary reagent".

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Einstein Center 3R – A joint initiative of Berlin research institutions

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In 2020, 2.533.664 animals were used for scientific purposes throughout Germany, of which 146.921 in Berlin alone [1]. The concentration of large research institutions with a strong focus on (translational) biomedicine in Berlin results in a high number of animal experiments. To reduce dependence on laboratory animals and to develop new models with a high degree of prediction, the focused development and consistent implementation of the 3R principle must be an integral task of academic biomedical research. The pressure to act is particularly high in Berlin, where a very lively research community meets quite committed animal rights activists.

Thus, the Einstein Center 3R (EC3R, ec3r.org) was launched. It is a joint initiative of major Berlin research institutions: Charité – Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, Technische Universität Berlin, Berlin Institute of Health, Bundesinstitut für Risikobewertung, Max Delbrück Center for Molecular Medicine, and Robert Koch Institute. The EC3R project is supported by the Einstein Foundation Berlin with funds from the Berlin Senate after it has gone through an independent review process. After a preparatory year, full funding started in July 2021.

EC3R is operating in three areas: "Research", "Education & Training", and "Communication & Outreach". EC3R aims to increase the trust in the 3Rs. Development of robust 3D tissue culture models, measures to increase scientific quality and transferability, comprehensive education in all the 3 Rs, and scientifically sound communication are essential parts of the activities and the strategy to reach this goal.

In terms of research, EC3R is focused on the development of alternatives to animal models, specifically the development of robust 3D tissue culture models. By means of a Berlin-wide call for proposals, 6 projects were identified (out of 28 proposals) to form the Collaborative Research Network. This network acts as a platform for overarching topics like agreements on standards and comparative validation of alternative methods. The network is complemented by two cross-sectional projects dealing with quality assurance and cross-species comparisons.

The education and training area primarily targets young scientists such as PhD students and postdocs. The goal is to build awareness for all 3Rs and substantial competence. In addition to the lecture and course offer and a call for Lab Visit proposals for all (Berlin) junior scientists, a special offer for young scientists within the EC3R is currently being established. It includes a mentoring program, an internal Lab Visit program, and other measures for exchange and networking.

Communication and outreach aim on the one hand at the scientific community and on the other hand at interested laypersons. The communication with the latter includes all 3Rs but focuses on the understanding of alternative methods. For any communication, emphasis is put on an honest and differentiated presentation, especially with regard to the opportunities and limitations of alternative methods.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Screening of vascular disruptors as an endpoint to predict developmental toxicity using zebrafish embryos

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The development of new blood vessels is a highly complex biological process. The proper function of the blood vessel system is needed to ensure an efficient blood circulation that takes care of the oxygen and nutrition supplies for the whole organism [1]. Therefore, it is not surprising that developmental toxicity can be caused by the disruption of angiogenesis. Perturbation of the blood vessel system results in severe birth effects up to lethality. Various chemicals have already been known to impact on in the disruption of blood vessel formation. These chemicals include herbicides [2] and insecticides [3] as well as pharmaceuticals [4]. There is a tremendous need to understand the mechanisms of angiogenesis in more detail, which provides a foundation to develop alternative screening tests avoiding the development of hazardous compounds and or prioritizing non-hazardous compounds and hence reducing animal testing in later stages. The alternative test model zebrafish embryo allows the screening for prenatal developmental toxicity given that it represents complex differentiating whole organism in a small-scale system. The major pathways for the development of blood vessels are highly conserved between vertebrates. A further advantage of zebrafish is the transparency of the embryos and visibility of circulating blood cells. Based on this feature, we have developed a label-free method to detect the vascular network in the wildtype zebrafish embryo, which enables a high-content screening for potential vascular disruptors. The visualization of the blood vessels and the representation of the inhibition of angiogenesis is achieved by video recording since the moving blood cells allow to map the blood vessel network in the tail region of the zebrafish embryo by subtraction of video frames. A widely used transgenic strain was included as a proof of concept. Furthermore, an automated, quantitative imaging-based assessment of morphology was conducted to compare the effects concerning angiogenesis to other phenotypic endpoints. Combining the transgenic strain with the subtraction images showed that even in case that sprouting events of vessels occur, no blood cells may flow through the vessels indicating already a functional impairment. This example highlights the added value of this technique for investigating the innovative endpoint of blood vessel inhibition as the method is label-free, and non-invasive, which captures the functionality of the blood vessels. Furthermore, quantitative assessment of angiogenesis inhibitors enabled the establishment of effect concentrations which were used to design transcriptome studies to assess underlying molecular processes. Both the video subtraction approach and transcriptome assessments were established with model angiogenesis inhibitor(s) (SU4312, SU5416, PTK787 and Sorafenib) that are known to interact with the VGEF receptor.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Bioprinting of organ models for infection studies

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The corona pandemic has underlined the urgent need to provide tools for the rapid development of antiviral strategies. Infection studies are particularly hampered by species-specific differences when using animal models. Many human-pathogenic viruses do not infect typical laboratory animals, or the course of infection substantially differs from that in humans. A possible solution for this issue is the use of 3D organ models that consist of human cells. For the generation of the tissue models, we use pneumatic extrusion bioprinters as well as a digital light processing (DLP) printer, which allow the precise positioning of cells in a given architecture.

In our initial lung model, alveolar epithelial cells (A549) were printed into a 3D grid-like structure in a bioink consisting of alginate, gelatin and Matrigel [1]. The model was successfully infected with influenza A virus. In our model, we observed a clustered pattern of infection, which is also found in the natural lung, but it contrasts with a 2D cell culture, in which infected cells are evenly distributed over the culture. Furthermore, the printed cells release interleukin-29 upon infection, which reflects a basic immune reaction.

To reflect the physiological function of the lung better, we further developed the lung model and printed a multi-cell type construct that consisted of a basic layer of primary human fibroblasts and THP-1 immune cells that were overlaid by a layer of the epithelial A549 cells [2]. Only in the multi-cell type model, A549 cells organized in cell clusters. The 3D tissue models were cultured for up to five weeks without substantial loss of cell viability. When challenged with a combination of the bacterial lipopolysaccharide toxin and ATP, an immune response was induced as shown by the release of proinflammatory cytokines IL-1beta and IL-8. The model was again infected with influenza A virus and a dose-response curve with the inhibitor Tamiflu was recorded, which demonstrates the usability of the model for the development of new antiviral drugs. We will now use the bioprinted model in a corona-research consortium, which relies on the use of animal experiments to evaluate the potential of 3D organ models to serve as an alternative to *in vivo* studies.

In parallel, a liver model was printed consisting of hepatocytes (HepaRG) [3]. In this model, extracellular matrix (ECM) from a human donor was used to replace Matrigel, which is obtained from mouse sarcoma and therefore constitutes a xenogenic component that is connected to suffering of animals. The cells in the model were found to express typical liver markers such as albumin and cytochrome P450 3A4 (CYP 3A4). The model was used to study transduction with an adeno-associated virus (AAV) vector of serotype 6, the most promising vehicle for gene therapeutic applications. The transduction efficiency was high as determined by the expression of a fluorescent reporter, and a short hairpin RNA expressed from the AAV vector efficiently inhibited expression of an endogenous gene (cyclophilin B). Furthermore, the model was shown to support replication of human adenovirus, which causes severe liver infections in immunocompromised patients. The liver model not only served as a tool to study virus infection but was also useful to investigate toxicity of cancerous agents [4].

Throughout our literature research, we became aware that most bioprinting studies (just like the majorities of all studies with stem cell or 3D tissue cultures) involve animal components such as murine basement membrane matrix, collagen, gelatin or fetal calve serum in the culture medium. This not only contradicts the aim of animal welfare, but it also produces chimeric systems with human cells culture in a xenogeneic environment. We thus propose strategies to replace these animal components by material of non-animal origin, an approach that we named *clean bioprinting* [5].

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Not applicable.

108

A serum-free medium that supports cultivation of fish cell lines: Case study about RTgill-W1 going serum-free

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The rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1, is found at the forefront of developing cell-based strategies and assay procedures that are intended to directly replace fish toxicity testing. For example, the RTgill-W1 fish cell line acute toxicity assay has been shown to accurately predict the acute toxicity of many chemicals to fish [1,2] and to do so robustly in academic and industrial labs [3]. On this basis, this assay has become the first of its kind to be approved by international standardization organizations, namely in 2021 by the Organization for Economic Co-operation and Development (OECD) as OECD TG249, and in 2019 by the International Organization for Standardization (ISO) as standard ISO21115. However, though the assay as such is performed in a simple buffer without the addition of animal components, e.g., to avoid interference with the chemicals or water samples to test, RTgill-W1 cells are grown routinely in Leibovitz's L-15 medium (L-15) supplemented with 5-10% fetal bovine serum (FBS) to enable cell proliferation. FBS is a very rich but undefined mixture of nutrients, hormones, etc., derived by unethical methods [4]. We therefore work to build a tailor-made, fully defined culture medium that would support the long-term cultivation of fish cell lines without FBS.

As a first step, we have developed a high-throughput 96-well plate RTgill-W1 cell proliferation assay and optimized it to enable systematic screening of serum-free media components, individually and in mixture. In this assay, the influence of selected supplements on cell proliferation is evaluated after 6 days based on cell metabolic activity (using the AlamarBlue assay), cell number (DNA staining with Hoechst 33342) and cell morphology (phase contrast imaging). The effects of the new media preparations on cell proliferation in L-15 with 5% FBS are benchmarked against cell number in L-15 alone, where cells are viable but do not proliferate. Using this method, we tested numerous inorganic and organic supplements, eventually selecting combinations that yielded metabolic activity, cell number and cell morphology closely resembling the routine culture conditions with 5% FBS.

The most suitable formulation mixture was then tested for long-term cell proliferation support in a second step by applying two serum-free adaptation strategies in parallel: a) sequential adaptation ("weaned cells") by gradual dilution of FBS with the serum-free medium and b) direct transfer of cells into the serum-free medium ("sink-or-swim-cells"). Both approaches were successful in adapting the RTgill-W1 cell line to serum-free medium. However, full adaptation was somewhat faster for "weaned" than for "sink-or-swim" cells: the former reached passages 3 and 10, which we set as benchmarks, more quickly than the latter. Currently, "weaned" cells are in passage 21 while the "sink-or-swim" cells are in passage 17.

Overall, the novel medium formulation enables RTgill-W1 cells to proliferate in the absence of serum for prolonged time. Fine-tuning of key supplement concentrations in the final formulation is now ongoing to allow for a fully optimized medium composition. Handling of RTgill-W1 clones in this new serum-free medium, including attachment, passaging, and freeze/thawing, is well underway. We may thus not be far from having the first fully defined culture medium for at least one fish cell line.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The role of physiologically-based kinetic models within the new approach methodologies framework for predicting cardiotoxicity

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The functional outcomes of cardiotoxicity are cardiomyopathy, arrhythmias and heart failure. The latter occurs when the function of the heart is compromised to the extent that it can no longer pump enough blood to oxygenate the organs of the body. Ultimately, this can lead to death. These functional outcomes cannot be measured *in vitro.* Instead, biomarkers of key events at the cellular and tissue level, which are associated with these outcomes, are measured [1]. The challenge is to understand how the resultant *in vitro* concentration-response curves can be interpreted to give meaningful predictions of adverse outcomes in humans, both on the individual and the population level.

Physiologically-based kinetic (PBK) models are deterministic computational models that use differential equations to capture and describe the biokinetics of chemical compounds. Their typical application is to predict the concentration of chemical compounds at their site of action at specified times following known exposures. PBK models range from comprehensive whole-body models to more detailed models of specific organs and tissues.

There is rising recognition of the value of PBK models within the new approach methodology (NAM) framework for predicting toxicity [2]. For example, they are used to translate the concentration-response curves that are measured *in vitro* to *in vivo* dose-response curves (iterative forward dosimetry) [3]. This is an essential step towards determining a Benchmark Dose (BMD) or a No Observable Adverse Effect Level (NOAEL) for regulatory purposes. They are also used to reconstruct *in vivo* exposures by combining biomonitoring data and *in vitro* concentration-response curves (reverse dosimetry).

In this presentation, we will examine how PBK models have been used in chemical risk assessment for cardiotoxicity. We will then proceed to discuss how these models can be refined given current knowledge of the adverse outcome pathways for cardiotoxicity, and the biomarkers that can be measured in advanced *in vitro* models.

This work was supported by MIUR-FIRB 2010 Future in Research (RBFR10L0GK), MIUR-PRIN 2010-2011 (2010J8RYS7), and the European Union's Horizon 2020 research and innovation program (101037090). The content of this publication reflects only the authors' view, and the Commission is not responsible for any use that may be made of the information it contains.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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

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The use of cell and tissue-based methods in basic, applied and regulatory science has been increasing exponentially. Animal-derived components, including serum, coating materials, growth factors and antibodies are routinely used in cell/ tissue cultures and in general laboratory practices. In addition to ethical issues, the use and production of animal-derived materials and reagents raises many scientific concerns, generally associated with presence of undefined components and batch-to-batch variability, which may compromise experimental reproducibility [1,2]. On the other hand, non-animal materials and reagents, such as human cells, alternatives to animal sera or non-animal recombinant antibodies, are becoming increasingly available, and their use is encouraged by the EU Directive 2010/63 and the Guidance Document on Good In vitro Method Practices (GIVIMP), published by the Organization for Economic Cooperation and Development (OECD) [3]. In an effort to map the current state of use of animal-derived reagents across different sectors and to identify the obstacles possibly hampering the implementation of non-animal derived alternatives, a global online survey addressed to scientists working on *in vivo*, *in vitro*, *in silico* methods, in academia as well as pharmaceutical or cosmetic companies, was conducted with the goal to understand: 1) the most commonly used animal-derived materials and reagents, 2) the main issues associated with the production and use of animal-derived materials and reagents, 3) the current level of knowledge on available non-animal alternative materials and reagents, and 4) what educational and information sources could be most useful or impactful to disseminate knowledge on non-animal alternatives. Here we provide an overview of the survey replies and discuss possible proposals to increase awareness, acceptance and use of non-animal ingredients.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Human lungs show limited permissiveness for SARS-CoV-2 due to scarce ACE2 levels but virus-induced expansion of inflammatory macrophages

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Background: SARS-CoV-2 utilizes the ACE2 transmembrane peptidase as cellular entry receptor. However, whether SARS-CoV-2 in the alveolar compartment is strictly ACE2-dependent and to what extent virus-induced tissue damage and/or direct immune activation determines early pathogenesis is still elusive.

Methods: Spectral microscopy, single-cell/-nucleus RNA sequencing, bulk RNA seq, qPCR, Western Blot, or ACE2 "gain-of-function" experiments were applied on infected human lung explants and adult stem cell-derived human lung organoids to correlate ACE2 and related host factors with SARS-CoV-2 tropism, propagation, virulence and immune activation compared to SARS-CoV, influenza and MERS-CoV. COVID-19 autopsy material was used to validate *ex vivo* results.

Results: We provide evidence that alveolar ACE2 expression must be considered scarce, thereby limiting SARS-CoV-2 propagation and virus-induced tissue damage in the human alveolus. In human alveoli as well as bronchial and alveolar organoids, SARS-CoV-2 replication strictly depends on ACE2, and adenoviral transduction of ACE2 in human lung tissue led to increased tissue damage. Expression of host factors such as ACE2, BSG/CD147, TMPRSS2, and FURIN remained stable in the different infections, with consistently low levels of ACE2. Instead, *ex vivo* infected human lungs and COVID-19 autopsy samples showed that alveolar macrophages (AM) were frequently positive for SARS-CoV-2. Single-cell/-nucleus transcriptomics further revealed non-productive virus uptake and a related inflammatory and anti-viral activation, especially in "inflammatory alveolar macrophages", comparable to those induced by SARS-CoV and MERS-CoV but different from SARS-CoV-NL63 or influenza virus infection. We found a significant depletion of the AM population together with an expansion of inflammatory AM both in lung explants and in acute autopsy samples. Antiviral and inflammatory pathways as well as apoptotic genes were upregulated in inflammatory AM. We compared expression patterns of the macrophage subpopulations identified here to published scSeq and snSeq data from lung autopsies as well as broncho-alveolar lavages. Besides the expected correspondences within more AM-like and more monocyte-like populations, we found high similarity between the inflammatory AM population identified here and some inflammatory macrophage subtypes described previously. Within this group, dataset-specific gene expression, regarding cytokine induction, indicates differences between lung autopsy tissue, BAL samples, and explant models at different stages of infection. In particular, we detected early induction of genes such as CCL3, CCL20, CXCL5, CXCL8 or IL1B in the inflammatory macrophages of the explant lungs, which seem not upregulated in later stages of infection in this macrophage subtype.

Conclusion: Collectively, our findings indicate that severe lung injury in COVID-19 likely results from a macrophage triggered immune activation rather than direct viral damage of the human alveolar compartment.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Development of 3D-printed surgical guides to standardize, refine and reduce animal experiments in osseointegration research (the "PRECISE" project)

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"PRECISE" is a highly interdisciplinary project that aims to standardize implant positioning as well as to reduce and refine animal use in osseointegration research. Funded by the Austrian Science Fund (FWF), this 3-year project started its activities in May 2022.

Medical bone implants, such as screws or artificial joints, play a major role in orthopedic surgery and dental medicine. Osseointegration, which is the formation of new bone at the surface of the implant as well as the functional connection between viable bone and the implant surface, is decisive for sufficient anchorage of the implant and its durability. Insufficient osseointegration may be caused by factors that affect bone metabolism (e.g., osteoporosis, smoking, diabetes) and may lead to mechanically induced osteolysis and, subsequently, to aseptic implant loosening. The rising demand for medical implants due to the world's aging population as well as the increasing number of implant failure and revision surgeries constitute a significant burden for healthcare providers and patients alike.

To study the process of osseointegration under experimental conditions such as osteoporosis, the rat tibia implantation model is used worldwide as the gold standard [1]. In this model, screws with a typical size of 1-2 mm are implanted into the proximal tibia of rats prior to histological assessment of osseointegration. It is widely accepted that a common source of errors in this model is the correct positioning of the implant, as it represents the most critical parameter to minimize the variability of outcome measurements. Besides a constant distance from the growth plate, equidistance from surrounding cortices and the insertion angle are essential. However, the flawless realization of these requirements is a major obstacle frequently resulting in failed experiments and an exaggerated number of used animals [1].

The "PRECISE" project aims to solve this scientific and ethical problem by use of 3D-printed surgical guides that exactly fit the shape of the rat tibia bone and allow exact positioning of the screws.

In a preliminary set of experiments, we confirmed the feasibility of critical elements of the proposed workflow. As such, relevant parts of the proximal metaphysis of a rat tibia bone were scanned using *ex-vivo* micro-computed tomography (μCT) imaging prior to rendering of image data files into a 3D file format using CAD software. Then, the surgical guide was digitally created and printed using non-biocompatible materials. Subsequently, we tested the practicability in a rat cadaver and learned that the guide was easy to handle and provided a tight fit that allows precise placement of the burr hole/screw bed. Analyzing the position of the screw using μCT revealed excellent positioning, both regarding the horizontal angle as well as the distance of the screw axis from the tibia lateral ridge, which exploited the maximum space of the intramedullary cavity

From this basis, the "PRECISE" project will design accurately fitting surgical guides for the tibia bones of three standard rat phenotypes. These guides will then be fabricated in plastic and metal using three different 3D printing technologies. The applicability of the guides is then evaluated independently by two surgeons in rat cadavers. The blueprints of the best performing guides will be provided to the worldwide community via databases. In a next step, a special imaging device (*in vivo* μCT) will be implemented into the workflow to allow 3D reconstruction of the tibia bones from living animals. Thereby, it will be possible to design individual surgical guides for practically every rat phenotype (different age, size, sex, disease context, bone morphology, etc.). Both approaches, the general and the customized design, will be compared in their practicability and implementation. Finally, the "PRECISE" project will for the first time systematically evaluate the impact of implant positioning (i.e., distance from the growth plate) on osseointegration. In this context, rats with compromised bone quality as well as the *in vivo* μCT technology will be used to assess the process of osseointegration *in vivo* over time.

In summary, the "PRECISE" project is expected to contribute to the standardization of the rat tibia osseointegration model, to refine the method and reduce the number of animals used in future scientific studies.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Attitudes towards animal dissection and animal-free alternatives among high school biology teachers in Switzerland

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Harmful animal use in education has raised ethical and environmental concerns as well as concerns about the potential psychological harm to students [1]. Consequently, there has been an ongoing debate during the last decades about the role and importance of animal use in teaching across all education levels. Animals are not only used in human or veterinary medicine training at universities but also as a part of general biology education in high schools. This tradition began in the early 1900s [2] and is still present in many countries [3]. The teachers' decision on whether to use animal dissection or alternatives can be influenced by many factors, for example, their own education, and/or previous experience with animal-free teaching methods [4]. The exploration of teachers' attitudes towards dissection and alternatives can provide a clearer picture of the barriers and opportunities for making the shift towards more humane biology education. Nevertheless, up to date, only a few studies investigated the attitudes and experiences of high-school biology teachers towards animal dissection.

This study aimed to evaluate, for the first time, the experiences, and attitudes of Swiss high school teachers towards the use of animal dissection and animal-free alternatives. Specifically, the survey intended to determine 1) the extent to which animals or animal parts are being used in biology classes in Swiss high schools, 2) whether Swiss high school teachers embrace and adopt humane teaching methods, and 3) the attitudes of teachers towards dissection and humane teaching methods.

We designed an anonymous online survey in which questions were organized into two parts: 1) a general part with questions about demographic data of the respondents, and 2) a scientific part with questions on the use of animal or animal organ dissection in their teaching practice. The survey contained a combination of open-ended questions and multiple-choice questions, allowing respondents to check one or more boxes from a list of possible answers. The first version of the survey was launched in August 2019 and an updated version with several additional questions was launched again in June 2021. A total of 76 teachers participated in the survey.

The vast majority (97%) of the teachers reported using animal / animal parts dissection in their classes. Mostly used are various animal organs, fish, insects, earthworms, squids, and rats or mice. As the obstacles to adopting alternatives were listed lack of time to research alternative methods, high costs, and peer pressure. The responses also revealed that a large proportion of the teachers are not convinced that animal-free alternatives are as good for teaching as the use of dissection. This is in stark contrast with the empirical evidence showing that humane teaching methods can provide equivalent or superior learning outcomes in comparison to the use of animals [5].

Our survey highlighted the barriers and opportunities for future work on raising awareness among high school teachers and the implementation of humane teaching alternatives. We conclude that the wider uptake of humane teaching methods would require financial support as well as a shift in the attitudes of high-school biology teachers. More widespread dissemination of information about available alternatives in particular might help teachers to adopt non-harmful practices and minimize the number of animals used in education.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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In vitro disease modeling of the Cockayne Syndrome B neuropathology using patient-derived iPSC – A 3R implementation approach

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It is widely acknowledged that animal models not always represent human disease. Especially human brain development is difficult to model in animals due to a variety of structural and functional species-specificities. This causes significant discrepancies between predicted and apparent drug efficacies in clinical trials, and their subsequent failure. Emerging alternatives based on 3D *in vitro* approaches, such as human brain spheres or organoids may in the future reduce and ultimately replace animal models. Here, we present a human induced pluripotent stem cell (hiPSC)-based 3D neural *in vitro* disease model for the Cockayne Syndrome B (CSB). CSB is a rare hereditary disease and is accompanied by severe neurologic defects, such as microcephaly, ataxia and intellectual disability with currently no treatment options. Therefore, the aim of this study is to investigate the molecular and cellular defects found in neural hiPSC-derived CSB models. Understanding of the pathology underlying CSB enables development of treatment options. The two CSB models used in this study comprise a patient-derived hiPSC line and its isogenic control as well as a CSB-deficient cell line based on a healthy hiPSC line (IMR90-4) background, thereby excluding genetic background-related effects. Neurally induced and differentiated brain sphere cultures were characterized via RNA Sequencing, western blot (WB), immunocytochemistry (ICC) and multielectrode arrays (MEAs). CSB-deficiency leads to an altered gene expression of markers for autophagy, focal adhesion and neural network formation. Cell migration was significantly reduced, and electrical activity was significantly increased in the disease cell lines. These data hint to the cellular pathologies possibly underlying CSB. By induction of autophagy, the migration phenotype could be partially rescued, suggesting a crucial role of disturbed autophagy in defective neural migration of the disease lines. Altered autophagy may also lead to inefficient mitophagy. Accordingly, disease cell lines were shown to have a lower mitochondrial base activity and a higher susceptibility to mitochondrial stress induced by rotenone. Since mitochondria play an important role in neurotransmitter cycling, we suggest that defective mitochondria may lead to an altered electrical activity in the disease cell lines. Failure of clearing the defective mitochondria by mitophagy, and thus missing initiation cues for new mitochondrial production, could potentiate this problem. With our data, we aim at establishing a disease adverse outcome pathway (AOP), thereby adding to the in depth-understanding of this multi-faced disorder and subsequently contributing to alternative drug development.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Evaluation of drug-induced liver toxicity of trovafloxacin and levofloxacin in a human microphysiological liver model

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Drug-induced liver injury (DILI) caused by already approved substances is a major threat to human patients, potentially resulting in the drug withdrawal and substantial loss of financial resources in the pharmaceutical industry [1,2]. To address the limitations of current drug testing strategies mainly involving two-dimensional cell cultures and animal testing, we leveraged a three-dimensional microphysiological model of the human liver sinusoid to investigate the toxicity of the withdrawn drug trovafloxacin and the structurally related non-toxic compound levofloxacin at human-relevant doses.

Liver sinusoidal models were successively assembled as described by Rennert et al. [3] within the Dynamic42 biochip. Therefore, upcyte® human liver sinusoidal endothelial cells (LSECs) and monocyte-derived macrophages isolated from human donor blood were incorporated into the top chamber to form a confluent vascular layer. The bottom chamber consisted of HepaRG™ hepatocytes. Unidirectional vascular perfusion, including the respective drug concentrations, was applied daily for a treatment period of up to 7 days. Afterwards, immunofluorescence staining and live cell staining were performed to survey cell-specific morphology and toxicity markers. Medium supernatants were collected from vascular and hepatic chambers and were analyzed for the release of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and released cytokines.

Trovafloxacin elicited vascular and hepatocellular toxicity associated with pro-inflammatory cytokine release already at clinically relevant concentrations, whereas levofloxacin did not provoke tissue injury. Similar to *in vivo*, cytokine secretion was dependent on a multicellular immune response, highlighting the potential of the liver model for reliably detecting drug-related cytotoxicity. Moreover, we elucidated hepatic glutathione depletion and mitochondrial reactive oxygen species (ROS) formation as intrinsic toxicity mechanisms contributing to trovafloxacin toxicity. Thus, by utilizing the liver model, we were able to reproduce trovafloxacin toxicity, which was not detected in mice studies without the addition of inflammatory co-stimuli [4].

In conclusion, our findings contribute to the understanding of immunomodulatory and intrinsic toxicity mechanisms of TVX and verified the non-DILI profile of LVX. Therefore, we emphasize the potential of our human liver microphysiological model as an *in vitro* platform for the evaluation of DILI. Our model could be relevant to address the limitations of conventional animal models and could prove useful to detect undesirable drug adverse events to avoid unnecessary animal suffering during preclinical testing.

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Submission declaration:
Conflicts of interest: M.R., K.R. holds equity in Dynamic42 GmbH. A.S.M. holds equity in and consults Dynamic42 GmbH.
Statement on ethics vote: The isolation of human peripheral blood mononuclear cells (PBMCs) from blood of healthy human donors was approved by the ethics committee of the Jena University Hospital (2020-1684, 3939-12/13). All donors were informed about the aim of the study and gave written consent.

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Animal-free plant-derived nanofibrillar cellulose hydrogels for cell-based assays with stem cell and organoid cultures

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The use of animal-derived hydrogels face challenges such as lot-to-lot variation and undefined consistency. Predictive, biologically relevant, and cost-effective *in vitro* cell models are needed for drug screening, disease modelling and other cell-based assays. Three-dimensional (3D) cell culture with hydrogels provides better *in vivo* relevancy, compared to traditional 2D monolayer culture.

GrowDex® hydrogels are plant-derived nanofibrillar cellulose (NFC) hydrogels which contains only nanofibrillated cellulose and water; and have been shown to provide an effective support matrix for culturing various cell types in 3D. Its shear thinning properties, no lot-to-lot variation, temperature stability, and tunable stiffness enable the use of NFC with many different cell types with reproducible results. As an animal-free matrix, NFC enables possibilities also for clinical applications.

NFC is a proven and effective tunable support matrix for cells, spheroids, and tissue excisions in 3D. These include for example different tissue organoids, stem cells and tissue excisions. (A) Renal organoids, primary mouse kidney metanephric (MM) cells, were cultured in NFC and chemically induced to undergo nephrogenesis. For these organoids, highly defined culture matrix is essential as many chemicals may distort the nephrogenesis process and NFC culture, now NFC allowed the organoids to grow in conditions mimicking better the natural physiological environment. The organoids showed no signs of toxicity, and the organoids grew more spherical compared to their control. Additionally, the nephrogenesis proceeded similarly to the control. (B) NFC has also shown to support mesenchymal stem cell (MSC) adipogenic and osteogenic differentiation potential when cultured in anionic NFC (aNFC) hydrogel. After 21 days of differentiation treatments in aNFC, the MSCs showed intracellular lipid accumulation and strong calcium deposition compared to the controls. MSCs require more relevant 3D physiological environment for differentiation and aNFC showed favorable environment to enhance the level of adipogenesis and osteogenesis of cultured MSCs. (C) Finally, NFC has shown also to be efficient and stable in supporting long-term cultures of *ex vivo* human biopsies of healthy skin, foreskin and glioblastoma samples at least for 28 days. Histological analysis of the samples revealed more stable maintenance of tissue integrity and sustained histopathological identities when compared to mouse sarcoma basement membrane extract (BME). Animal-derived culture materials are known to affect the biopsy environment introducing xenofactor based bias and affecting cell behavior through animal proteins and growth factors. These results indicate the importance and need of robust and defined animal-free 3D biopsy culture model for more realistic disease modelling, phenotypic screening, tumor microenvironment analysis and high throughput 3D drug screening.

The key to developing biologically relevant, predictive, and cost-effective *in vitro* cell and *ex vivo* tissue models is repeatability with minimum variation between experiments, and *in vivo* relevancy with a suitable *in vivo* like environment. This can be done with the use of automation, automated liquid handling, and bioprinting systems. GrowDex hydrogels physically resemble the extracellular matrix, allowing free diffusion of small molecules such as nutrients and oxygen. These properties make NFC based GrowDex hydrogels suitable matrices for animal free *in vitro* applications. Authors would like to thank Ulla Saarela and Johanna Niklander for performing the experiments.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Enhanced drug metabolisms of hepatocytes with physiological aerobic respiration enabled by a new oxygen-permeable plate as an alternative culture format

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In pharmaceutical industry, cultured primary hepatocytes are one of the standard tools to assess hepatic metabolisms and toxicity *in vitro*. Drawbacks include their functional deterioration upon isolation due to the lack of physiological environment. Specifically, the very low oxygen supply flux via culture medium layer in standard tissue-culture-treated polystyrene (TCPS) in static condition was pointed out over 50 years ago [1], but it is not well recognized even now. Polydimethylsiloxane (PDMS) has been reported to enable aerobic respiration of cultured hepatocytes and thus enhance the metabolic functions by its high oxygen permeability [2] and are often used as a material of microphysiological systems (MPS) [3]. However, its high chemical sorption property retarded its practical use in drug development.

As an alternative material to PDMS, we evaluated a new culture material, 4-polymethyl-1-pentene polymer (PMP). This is an olefinic polymer that express high oxygen apparent permeability comparable to that of PDMS when fabricated with thin films. First, using primary-cultured rat hepatocytes, we confirmed high oxygen consumption of rat hepatocytes in PMP as can be observed in PDMS and low drug sorption property of PMP comparable to those of TCPS. Then, we confirmed that high levels of liver function including various drug metabolisms were maintained at least for a week as well as remarkably enhanced albumin production. Interestingly, productions of some of the drug metabolites were remarkably enhanced in PMP when compared in PDMS or TCPS.

These results on the new PMP-based culture plate show that it enables more accurate evaluation of drug metabolism profiles due to the combination of high metabolic functions of cultured hepatocytes and low sorption of original drug and its metabolites. As such, a new material, PMP, is expected to replace PDMS in variety of drug testing systems ranging from static plates to various types of MPS devices.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Animal and tissue exchange platform (ATEX)

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A significant proportion of the experimental animals that are redundant are generally killed. These are animals that have been used for breeding, animals that have been bred but for which there is no scientific purpose, or animals that are alive and healthy after use in an experiment. These healthy surplus laboratory animals can possibly be used in new experiments, for which no animals need to be purchased or bred.

In addition, laboratory animals (and sometimes other animals) are also used as tissue donors in *ex vivo* and *in vitro* research. This often involves only one organ or piece of tissue. But often there is no destination for the remains and these are either destroyed immediately after collection or left unused for years in researchers' repositories. If fellow researchers know in good time which tissues and organs are or will become available, and if researchers inform colleagues in good time about which organs or tissues they need, it is possible to make better use of laboratory animals.

The Animal and Tissue Exchange platform (ATEX, https://atex.uu.nl/) is currently available at Utrecht University and University Medical Centre Utrecht. It makes supply and demand of animals, organs and tissues visible and thus contributes to their better use. ATEX is intended for the exchange of:

- Live surplus laboratory animals
- Fresh animal organs and tissue
- Preserved animal organs and tissue

As a result, it is expected that fewer laboratory animals will be needed overall.

This platform is developed by the Animal Welfare Body Utrecht and the 3Rs Centre. It is provided and maintained by the AWB Utrecht.

It is anticipated that also other universities will be using ATEX in the near future.

A demo version is available at: https://demo-atex.uu.nl/

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Towards 3R: X-ray fluorescence imaging as a novel non-invasive tool for longitudinal pharmacokinetic studies

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X-ray fluorescence imaging (XFI) is based on the excitation and subsequent detection of X-ray fluorescence photons excited by a scanning incident X-ray beam. In order to make entities of interest visible, they need to be labelled with markers such as molecular iodine or palladium nanoparticles. The spatial resolution of the modality is solely determined by the applied X-ray beam diameter, which is typically in the range between 0.2 and 1 mm for *in vivo* measurements and between 80 and 200 nm for single cell measurements [1].

As the modality is non-invasive and the used markers do not decay over time, longitudinal studies are possible in one and the same animal, hence offering the potential for a significant reduction of test animals. Moreover, not only a single marker element, but multiple ones can be tracked simultaneously, providing the possibility to monitor multiple entities such as different subtypes of immune cells or medical drugs in one and the same test animal. Applications comprise, among others, uptake measurements of functionalized nanoparticles in tumor cells [2] or mRNA-carrying nanoparticles, monitoring of T-cells in cell therapy [3] or in immune-mediated inflammatory diseases. Recently, we have published a quantitative proof-of-concept experiment, demonstrating the high sensitivity at high spatial resolution by measuring the spatial distribution of the endogenous iodine content in a murine thyroid [4].

This talk will cover the physical principles of the modality, presenting results of recent pilot studies and discussing different application fields of XFI, especially in the context of 3R.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

120

The Innovation Center for 3Rs (IC-3Rs) in Belgium

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Creation of IC-3Rs: Since the introduction of the three Rs Principle (Replacement, Reduction and Refinement), attitudes, laws and practices related to the use of laboratory animals in education, research and testing have undergone important changes [1]. Among other developments National Centers were created to contribute to the implementation of the 3Rs Principle. The first activities in Belgium date back to the late 1980s and 1990s, under the auspices of HRH Prince Laurent. Initiatives taken by young researchers at the "Vrije Universiteit Brussel" (VUB) at that time resulted in the foundation of the "Belgian Platform for Alternative Methods" (BPAM) which developed further into the scientific association "European consensus platform for alternatives" (ECOPA). In fact, it can also be seen as the predecessor of the actual "Innovation Center for 3Rs" (IC-3Rs) at the VUB which was officially founded in September 2017 within the research group of *In Vitro* Toxicology and Dermato-Cosmetology (IVTD) [1].

Goal of IC-3Rs: IC-3Rs is a scientific platform that promotes the implementation of the 3Rs in research and education by using state-of-the-art technologies and informing relevant stakeholders about them. In addition to advancing human health, it also contributes to improving animal welfare. The ultimate goal of IC-3 is to replace animal tests by alternative methods whenever scientifically sound and evidence-based.

Structure of IC-3Rs: IC-3Rs is currently managed by Prof. Vera Rogiers, Chairholder of the Chair Mireille Aerens at the VUB, and is guided by an international Steering Committee and a communication officer. The activities of IC-3Rs are supported by the legacy of the philanthropist Mireille Aerens and the Animal Welfare Department of the Brussels Region (Former Cabinet of Bianca Debaets and current Minister Bernard Clerfayt).

Activities of IC-3Rs: (i) to provide research opportunities to young PhD students to develop and use New Approach Methodologies (NAMs); (ii) to organize annual symposia on 3R-related topics; (iii) to stimulate collaborations with scientists, public research institutes, industry, funding organizations, regulatory authorities and animal welfare bodies and (iv) to disseminate up-to-date information on NAMs via the IC-3Rs website (https://www.ic-3rs.org) and the RE-Place open access database (https://www.re-place.be). The latter is supported by the Flemish and the Brussels Region. It is a unique database, coordinated with Sciensano, which aims to centralize all available knowledge on NAMs in Belgium and provide the names of scientists and institutes with expertise in these (new) methodologies. IC-3Rs is also partner of the recently approved COST project IMPROVE which establishes a 3R-network that will work to refine, harmonize and promote the 3Rs Principle, data and documents, in order to improve the quality of biomedical science [2]. Furthermore, IC-3Rs is involved in the EU projects ONTOX, Twinalt and PARC and in several national research projects, all dedicated to the development and use of alternative methods in the broadest sense.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Outcomes from a multistakeholder engagement to address the challenges associated with the development, regulatory approval, and implementation of non-animal methods in safety testing and biomedical research

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Animal use in research has its origins in antiquity. The rigor, reproducibility, and translatability associated with that use, however, is a modern concern. While the complementary ARRIVE 2.0 and PREPARE guidelines provide a path for accurate reporting, and properly performed systematic reviews aid in selecting animal models, none of these assistive research tools ensures an outcome perfectly mimicking human physiology or pathophysiology. In the case of safety testing, efficacy of a drug in animal models has often lacked correlation to human consequences, leading to harmful outcomes. Clearly, there is a need to develop models with improved predictivity. Non-animal methods, also referred to as NAMs, are increasingly being considered as adjunctive investigative tools, or, in some cases, full replacements for the use of animals in safety testing and biomedical research. This presentation will report the outcomes from two broad-based stakeholder conversations that deeply dissect the major challenges associated with the development, regulatory approval, and implementation of NAMs in safety testing and biomedical research. These conclusions support the collaborative design of reliable pathways toward the human-relevant methodologies which will usher in a new era of enlightened, humane, and results-oriented science.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Advanced *in silico* approaches for designing human-relevant organ models

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Introduction: In silico methods provide a powerful support for studying and optimizing artificial organ systems, such as organoids and assembloids [1]. In fact, the plethora of parameters conditioning organoid growth and morphology can be explored in virtual models, facilitating experimental design, and enabling prediction and extrapolation of behavioral and functional traits. Here we combine statistical physics and computational intelligence to predict how cells in computer-generated constructs cooperate to share resources (e.g., oxygen), and how their morphology and function emerge.

Methodology: Kleiber's Law (KL) is a universal law of biology, which states that the metabolic rate of an organism scales with its size according to a quarter-power law [2]. Its pertinence for designing human-relevant *in vitro* models has been highlighted, and computational models have been implemented for identifying the size range in which three-dimensional (3D) cell constructs obey KL [3]. However, KL is formulated as a deterministic framework, although fluctuations and heterogeneity are inevitable and known to shape the response of biological systems to external perturbations (e.g., nanoparticles, viruses). In this light, we implemented finite element models for investigating oxygen metabolism in simulated cell constructs of different sizes, considering stochastic variability for morphological (e.g., shape) and functional (e.g., kinetic constants) parameters. We generated joint distributions of construct masses and metabolic rates, developing new statistical tools to test whether and in which construct size range a generalized stochastic formulation for KL applies [4]. KL is then implemented along with other physical, metabolic and mechanical constraints using a computational intelligence-based method, so as to define optimal design criteria for functional models in terms of cell construct sizes and shapes. Specifically, novel evolutionary algorithms based on the minimization of a cost function incorporating biophysical constraints – e.g., resource uptake, surface energy minimization, cooperative metabolic effort – enable the generation and iterative reshaping of model input datasets.

Results: We found that stochasticity significantly restricted the range of cell construct sizes complying with KL, implying that, to date, many cellular models may lack translatability [5]. Ongoing experimental validation – integrating high resolution optical sensors, algorithms for multiparametric fitting and advanced imaging – suggests that 3D cell constructs manifest cooperative metabolic behaviors and that variability and fluctuations of morphological and functional characteristics confer robustness to biological systems.

Conclusions: In silico models are crucial to the definition of robust criteria for designing human-relevant organ models, i.e., with enhanced predictive and translational value. Used to optimize experimental design, they can substantially reduce development costs of *in vitro* constructs, representing a key step towards a reliable alternative to animal tests. The potential of computational and mathematical models has yet to be fully exploited though. Given the acceleration of computational power – even in basic machines – classic *in silico* methods integrated with artificial intelligence (e.g., evolutionary and genetic algorithms) such as described here, will be able to produce robust high-throughput datasets which are unachievable *in vitro*. In this perspective, *in silico* models may gradually emerge as a potential cost- and time-effective replacement of both *in vivo* and *in vitro* approaches, paving the way to a more consistent, accessible and sustainable research in life sciences.

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Submission declaration: This work has received funding from the Swiss National Science Foundation, through Project SINERGIA 2019 572 CRSII5_186422/1.
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Latest activities of JSAAE and Asian organization

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After the establishment in 1989, the Japanese Society for Alternatives to Animal Experiments (JSAAE) [1] has been promoting 3Rs research in Japan through a wide variety of activities in versatile areas not only cosmetics but also pharmaceutical, food and chemical industries. In terms of applications of new technologies, we have been involved in national projects such as AI-based hazard prediction [2] (2017-2021) and MPS for pharmaceuticals developments [3] (1st phase, 2017-2021; 2nd phase, 2022-2026). Simultaneously, we have been developing international cooperation with EUSAAT, ESTIV and ASCCT as well as Asian counterparts. The next step is to establish Asian Federation in alternatives research.

Previously, toward the better Asian organization, we initiated the Asian Congress series in Karatsu, Japan, in 2016, which was continued by Chinese Society in Guangzhou, China in 2018, and then will be hosted by KSAAE in this year, December 14-16, 2022, in Cheju Island in Korea [4]. Primary mission of Asian Federation is to support this series of regional conferences. JSAAE, KSAAE or TATT (Society of Toxicological Alternatives and Translational Toxicology, Chinese Society of Toxicology) and TTAM (Society of Toxicity Testing and Alternative Methods, Chinese Environmental Mutagen Society), strongly wish to have such Federation, as well as other new Asian societies such as SAAE-I (Society for Alternatives to Animal Experiments-India) and SAAT-SL (Society for Alternatives to Animal Testing in Sri Lanka) do.

Currently, we are discussing by-laws and possible organization structure of the Asian Federation among. We strongly hope that we can have a signing ceremony in coming 3rd Asian congress in this December in Korea for the establishment of the Asian Federation. Another important mission is to establish an official scientific journal of the Asian Federation. This is strongly expected by Asian alternatives researchers. Also, we highly recognize the importance of young generations and their communication across the Asian region to ensure the sustainable development of alternatives research and their social implementations in this area. We believe these directions ensure the sustainable 3Rs development both in Asian countries and the worlds.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Animal components in life sciences and their alternatives

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Currently, great effort is being made to develop alternatives to animal experimentation. New approach methodologies (NAMs) make continuous progress and aim at replacing conventional *in vivo* approaches. Among the most promising developments are stem cell research, organoid culture, bioprinting of organ models and multi-organ-chips. It is important to note that alternatives to *in vivo* tests will not only contribute to the aim of increasing animal welfare, but it will also produce knowledge with higher relevance to the human (patho-)physiology, as many results from animal experiments cannot be transferred to the human patient due to species-specific differences. However, despite the progress made through the development of NAMs, these technologies are usually based on well-known as well as hidden animal components. We have recently analyzed the extent to which animal products are used in bioprinting technologies [1]. Thus, technologies aiming at contributing to the 3R principles are sometimes causing substantial suffering of animals and lead to questionable results by producing chimeric systems.

A well-known animal product used in virtually all types of eukaryotic cell culture is fetal bovine serum (FBS), which is harvested from unborn calves of slaughtered cows. While the suffering connected to this procedure is under debate, the use of FBS in cell culture has several unquestionable disadvantages: It produces a chimeric system by culturing human cells in bovine media, the outcome of experiments may vary due to batch-to-batch variations, and the high content of growth signals in the fetal serum supports the desired rapid proliferation of cells, while at the same time producing an artificial situation as the normal physiology is not always associated with rapid cell division. Two main alternatives to the use of FBS as a media supplement exist, either the use of human platelet lysate or the development of chemically defined media. Although these alternatives are well-known, hardly any lab substitutes the use of FBS. Reasons for these difficulties will be discussed.

In addition to FBS, Basement Membrane Extracts (BME), widely known under the brand names Matrigel and Cultrex, are commonly used in 3D cell culture. In this case, awareness still must be generated that these extracts are harvested from mice that were implanted Engelbreth-Holm-Swarm sarcoma. Alternatives include the use of human extracellular matrix or recombinantly expressed collagens.

Furthermore, many standard methods in biochemistry and molecular biology rely on proteins derived from animals. For example, antibodies commonly obtained from mice or rabbits are used for Western blotting, immunohistochemistry, flow cytometry and numerous further applications, trypsin from porcine pancreas is normally used to detach cells for passaging, and collagen from rat tails, pigs or cows and gelatin are important components for hydrogels, which are, for example, used for bioprinting. For most of these animal materials alternatives exist but are not commonly used.

Substitution of animal materials in biomedical research is usually hampered by the lack of time to establish alternatives and their higher cost. In addition, results from experiments using the standard methods with animal-derived materials constitute the gold standard in the published literature and the use of non-animal alternatives can cause difficulties in getting deviating results accepted.

NAMs devoid of animal components will contribute to animal welfare. However, to become commonly accepted, it will also be necessary to demonstrate that they generate results with higher relevance to the human biology.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Not applicable.

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Perspectives and challenges of use of human placenta-derived biomaterials in cell culture and regenerative medicine

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Introduction: There is unmet need for representative *in vitro* diagnostics for various applications, or functional human tissues to support or replace injured tissues or organs *in vivo*. While many synthetic and natural non-human materials were already established for use in 2D and 3D applications, they still do not mimic the complex functions of the sum of the extracellular matrix (ECM) in native, intact human tissue [1-3]. Here, we describe strategies to isolate extracellular matrix proteins from human placenta to develop a platform technology for applications in cell culture and regenerative medicine. Atelocollagen-I and other subtypes were isolated by pepsin digestion followed by salt-precipitation and further purification steps. Moreover, HUMAN PLACENTA Substrate® (hpS), a liquid mixture of various ECM proteins, and native laminin-111 were obtained by a Tris NaCl buffer extraction and further processing [4,5].

Methods: The products were characterized by various photometric or antibody-based methods. 2D and 3D *in vitro* cell culture experiments have been performed by using different cell types.

Results: HUMAN PLACENTA proteins were extracted for potential industrial processing. In 2D *in vitro* experiments, with human ECM coating cells showed a higher viability rate when compared to bovine or porcine materials. HUVEC cultured in a mix of hpS and fibrinogen showed randomly oriented 3D cell networks, which were formed after approximately one week of culture. Organoids of colon cells isolated from malignant carcinomas were grown and HepG2 were successfully bioprinted using a mix of hpS with alginate.

Discussion and conclusions: We have established effective methods to isolate multiple proteins with bioactive properties from human placenta tissue for various potential applications in tissue engineering and regenerative medicine (TERM). These materials can be used as a novel human-material-based platform technology in various 2D and 3D *in vitro* assays, for, e.g., 3D bio printing, cancer or toxicity studies, and probably also for *in vivo* applications. However more research is necessary to assess the full potential of this HUMAN PLACENTA platform technology for TERM.

Financial support was received from the Austrian Research Promotion Agency FFG (# 849755).

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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

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Glioblastoma (GBM) is the most prevalent primary intrinsic brain tumor and amongst most lethal forms of cancer with a median survival of 14.6 months. GBM tumors exhibit vast inter- tumoral and intra-tumoral heterogeneity, complicating the development of effective therapeutic strategies. Studies suggest the presence of self-renewing, tumor-propagating cancer stem cells (CSCs) in GBM tumors plays a crucial role in the resistance to conventional therapies by multiple mechanisms, including increased DNA repair [1]. First line treatment includes surgical removal followed by temozolomide chemotherapy and radiation.

Current standard model are patient derived xenograft (PDX) models. These models have limited ability to recapitulate the tumor microenvironment in GBM, even when transplanted into the central nervous system (CNS), limiting their use in drug development. Other methods, such as CNS implantation of glioma stem cells or neurospheres in nude mice generates brain tumors that preserves tumor heterogeneity. Yet, both approached fail in translation, as several drugs discovered in these models, and showed favorable results in pre-clinical studies using CNS PDX, have failed in clinical trials [2].

Recent studies describing three-dimensional organoid culture methods recapitulate features of *in vivo* cell growth, allowing self-organization, differentiation, and heterogeneity to exist within these tumors, unlike traditional spheroid cultures that present limited cellular heterogeneity and tumoral niches [1].

In this study, we present a method for creating vascularized multi-zonal patient-derived GBM organoids by precise robotic deposition. Precise deposition of CSCs and endothelial cells in a confined microenvironment creates a complex tumoral heterogeneity and vascularization. These patient-derived GBM organoids self-organize and allow three-dimensional cellular interactions, unveiling great cellular diversity and spatially defined cell phenotypes that are not seen in typical spheroid cultures.

Investigation of the organoids reveal multiple tumoral niches. An outer rim that inhabits a nutrient-rich, Ki67 positive proliferative niche that resemble the leading niche of clinical tumors. An inner core that displays senescent and quiescent cancer cells that mimic the clinical tumor hypoxic niche. CSC-rich niche localized around vascular networks that features aspects of the clinical perivascular tumor niche. Interestingly, patient-derived organoids, generated from treatment-unresponsive patients CSCs exhibit similar resistance to treatments, in oppose to spheroid cultures.

The method also allows the incorporation of oxygen micro-sensors in the tumor organoids in high content screening 384-well setups with minimal variability. We show that sensor-embedded patient-derived organoids can be maintained and kinetically monitored for several days under controlled physiological conditions, allowing treatment-relevant high throughput screening.

Kinetic screening of 126 FDA approved anti-cancer drugs in 2 different patient-derived organoids uncovers uniquely distinguished response patterns among different cancer treatments. Some drugs damage tumor cells but permit recurrence after several days, other drugs damage the tumor rapidly but show high toxicity in other tissues. Surprisingly, the slow acting drugs that are commonly missed in standard screens due to their delayed effect, seem to damage the roots of the cancer, and CSCs particularly, while exhibiting reduced toxicity in neighbouring tissues.

Network analysis of the assay's responses reveals several novel pathways that shed light on basic mechanisms underlying the glioma stem cell responses to anti-cancer treatment and enabled us to delve into new targets that could specifically damage CSCs. Our platform offers a new approach of kinetic high content drug development for complex tumors, providing valuable information for both novel drugs and repurposing attempts.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Preregistration in animal research – Animal welfare and scientific progress

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In recent years, it became increasingly evident that the 3R principle might not be sufficient to fully address ethical concerns about animal experiments. The use of animals for scientific purposes in our society is only tolerated under the basic assumption that the knowledge gained will advance research in crucial areas. This implies, that performed experiments are reported in a way that enables peers to benefit from the collected data. However, recent studies suggest that a large proportion of animal experiments are actually never published. In addition, poor study design, sketchy reporting, and questionable research practices like HARKing (hypothesizing after results are known) and p-hacking strongly impair the validity of published research. This not only slows down the scientific progress it also represents an ethical issue if animal lives are wasted without bringing any knowledge gain.

We propose preregistration of studies involving animals as a powerful tool to encounter questionable research practices and to increase transparency in biomedical research. Thoroughly planned studies, a clear distinction between planned and unplanned statistical analyses as well as reporting of all experiments conducted can improve the validity of preclinical research outcomes and can reduce the number of animal experiments in the end.

We have developed animalstudyregistry.org, an online platform for the preregistration of animal studies conducted worldwide [1,2]. It supports researchers in planning their study by asking detailed questions about the study design, methods, statistics and the animals used. The author of a registered study has the possibility to choose an embargo period for up to five years during which the study appears in the registry only with the title, name of the institution, short abstract and optionally the name of the author. With registration each study receives a DOI (Digital Object Identifier) making the study plan citable. As a federal institute, the Bf3R can guarantee continuity, data security, and independency of sponsorship.

Three years after the launch of the platform, the number of preregistrations is still low. Beyond informing researchers about the possibility of preregistration for animal science and the benefits for their research, we have to convince stakeholders to incentivize open science practices. Contributing to transparency has to become a value of its own, which needs to be rewarded by research institutions, funders, publishers, and regulators [3,4].

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Ex vivo human precision-cut (tumor) lung slices as a tool for the evaluation of CD109 in non-small-cell lung cancer

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Although *in vitro* cancer cell lines and *in vivo* animal models have many advantages, they go hand in hand with several well-known limitations. Alternatively, human precision-cut (tumor) lung slices (PCLS/PCTLS) can be used. These tissue slices preserve the original 3D microanatomical architecture of the human lung and ensure the retention of all immune cells in their complex and native microenvironment. As such, these human tissue slices represent an ideal *ex vivo* organotypic model for analyzing distinct immune cell populations and target validation in cancer, making additional *in vitro/in vivo* experiments redundant.

Non-small cell lung cancer (NSCLC) remains a leading cause for cancer-related deaths worldwide [1]. Due to oncogenic mutations and resistance development, there is a high need for novel targeted patient-based therapies. The glycoprotein CD109 is discussed to contribute to tumorigenesis and presents a highly appealing target for anti-cancer therapy [2]. Herein, we apply a primary human *ex vivo* setting to identify cell types that overexpress CD109, and to elucidate its specific role within lung cancer.

In order to acquire PCLS/PCTLS, fresh human lung tissue was obtained from cancer patients undergoing surgical resections. The received lung tissue was filled with agarose and put on ice to allow polymerization. Thin tissue slices could be generated from both the tumor site (PCTLS), as well as from the healthy tissue site (PCLS), within the same donor. Expression of CD109 was visualized in both PCLS and PCTLS using confocal microscopy. Utilizing flow cytometry, CD109 expression was also determined on macrophages isolated from both tumor (i.e., tumor-associated macrophages, TAMs) and adjacent, non-tumorous human lung tissue. In addition, CD109 protein levels were assessed by western blot in *in vitro* differentiated macrophages. These M1 and M2 macrophages were obtained via magnetic isolation of monocytes from peripheral blood mononuclear cells (PBMCs) using CD14 microbeads, and by further differentiation using specific cytokines and growth factors. The phenotypic identity of all acquired macrophages was confirmed by their phagocytic capacity, via cytokine level assessment (IL10, IL12, TNFα), and via flow cytometric surface (CD45, CD86) and intracellular (CD 206, CD163) marker stains.

Immunohistochemical comparison of tumor PCTLS and donor-matched non-tumorous PCLS confirmed a higher expression of CD109 in the tumor tissue, mainly showing surface expression on clusters of small cells as well as on larger single cell types. Preliminary data revealed a higher expression of CD109 on TAMs isolated from the tumor site, in comparison to donor-paired non-tumoral macrophages (55.5% vs. 16.9% $CD45^{+}CD109^{+}$ cells respectively). Moreover, CD109 was shown to be differentially expressed on *in vitro* differentiated macrophages, displaying a higher protein expression on macrophages with an M2 phenotype in comparison to M1 macrophages.

Our results confirm overexpression of CD109 in tumor PCTLS and reveal an increased expression on both *ex vivo* TAMs and *in vitro* differentiated M2 macrophages. We hypothesize that CD109 supports the M2 phenotype, and thereby possibly creates a pro-tumorous microenvironment. Altogether, healthy PCLS and tumor PCTLS represent a versatile tool for target validation. Moreover, CD109 can be considered as a promising target for immune-modulatory therapy of NSCLC.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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In silico methods: Empowering and mainstreaming "alternatives"

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Alternative or non-animal methods generally make use either of *in vitro* experimental models (spheroids, organoids, organs-on-chip, tissue-engineered models) or *in silico* models (computational or mathematical models). *In silico* and *in vitro* tools may potentially interact through the development process of alternative methods. For instance, computational models can support the design of engineered tissues to achieve target mechanical or diffusion properties. Likewise, *in silico* methods can preselect drugs to be screened experimentally in *in vitro* models for their preclinical assessment.

One of the biggest challenges for the integration of non-animal methods into mainstream basic and applied biomedical research is the demonstration of their translational value. The establishment of new synergic interactions between *in silico* and *in vitro* tools is necessary to address queries in regulatory science and for them to be embraced as a valid, stand-alone research tool. For example, newly designed *in vitro* human tissue models are generally validated experimentally, based on their cell and extracellular matrix composition, structure, functionality and response to control treatments (e.g., the administration of drugs with well-known clinical effects). Successively, the translational potential of *in vitro* assays making use of such new *in vitro* experimental models could be quantified and scored through mathematical tools and models – exploiting novel approaches based on machine intelligence – enabling extrapolation, prediction and risk assessment.

A major obstacle in the bridging the gap between *in vitro* and *in silico* models is the lack of scientists with interdisciplinary expertise in experimental and computational modelling and the difficult interactions between experts from the respective fields. Most experimentalists consider mathematical models, or more in general *in silico* models, as esoteric, complex, and intractable: they find them difficult to understand and apply. On the other side, *in silico* modelers often struggle to find key parameters or adequate datasets to generate meaningful outcomes, useful for example in gaining mechanistic insights into experiments. The barriers currently existing between *in vitro* and *in silico* models – and modelers – should be broken down to obtain a cross-contamination of tools and methods.

How can we bring the two areas closer to accelerate the development of better technology, give the models scope for improvement and optimization and so widen the uptake of advanced *in silico* methods such that they become the conventional rather than "the alternative"?

The aim of this talk is to present the landscape of *in-silico* models in a way that renders them accessible to experimental scientists, paving the way to a sustainable dialogue and the creation of new integrated paradigms. I will provide an overview on traditional *in silico*/mathematical models: models which describe the concentration versus time profiles of substances in organ and tissue compartments through differential equations (such as physiologically based pharmacokinetics – PBPK, clearance, nutrient diffusion and reaction), receptor-ligand models, mechanical models of cell and tissue assembly, and statistical methods based on comparison, classification and pattern recognition (often used for analyzing molecules with potential risks according to their structure, as in QSAR – quantitative structure-activity relationship). I will also cover new methods based on artificial intelligence approaches, such as machine learning, data mining, fuzzy logic and genetic algorithms, focusing on specific applications such as cell identification, parameter optimization *in-vitro* to *in vivo* extrapolation (IVIVE) and distribution mapping.

Finally, I will present a couple of examples of how *in silico* methods are employed to support or reinforce experimental data from *in vitro* or *in vivo* methods, or to design experiments with better predictive or translational potential.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Innovative models in biomedical research

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Animal models are traditionally used in biomedical research to recapitulate human disease features and develop new drugs, as they are generally supposed to mimic some of the major hallmarks of human diseases. However, animals' models do not develop the disease as it occurs in humans, and their use has not supported efficiently the development of drugs for many highly prevalent non-communicable diseases, such as Alzheimer disease [1]. Indeed, despite conspicuous investments in research, the clinical failure rate in drug development remains very high. At the same time, enhanced clinical trials as well as surrogate human tissues, advanced imaging modalities and human epidemiological studies, may facilitate the development of safe and effective pharmacologic interventions [2].

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 was 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.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Promoting 3Rs public awareness in a young audience

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The foundation for children's future development is laid early and this foundation provide a strong base for long-lasting learning activities, cognitive abilities, and social interactions. Therefore, educating a young audience is key to stimulate critical thinking and changing mindsets of future generations, and for this reason, we believe that increasing awareness of actual animal experimentation circumstances and its alternatives is key to better understanding of science and to stimulate a younger generation in ethical thinking in the evolving human-animal relationship of the future.

This project has brought together a wide range of actors – the Swiss 3Rs Competence Center, EU Commission's Joint Research Center, Center for Alternative to Animal Testing, Altertox, Frontiers for Young Minds, and academic partners – all with the joint ambition to provide teachers with tools necessary to educate young minds about the intricacies of animal experimentation and how we can work to replace them.

The goal of this project is to create awareness among secondary school kids about animal experimentation and the importance of the 3Rs Principle, as well as providing teachers with material to implement teaching of this topic in schools. The project consists of three main elements – a workshop for teachers and educators on how to teach about 3Rs, an event involving three middle-school classes (11-12 years old) receiving lectures from scientists on different angles of the 3Rs, and finally, the same three classes will review three lay articles written by the speakers of the event and these articles will be published in the scientific journal for kids, Frontiers for Young Minds (kids.frontiersin.org). A mini documentary featuring the concept of the project has also been captured in interviews with the children and project stakeholders, and with this we hope to inspire others as well as to promote the 3Rs to a broader audience and generate social awareness around the topic.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Fish cell lines as animal-free and resource-efficient alternatives to fish in environmental risk assessment

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Millions of fish are used every year in the safety testing of chemicals and water samples. Alternatives to these animal experiments are urgently sought for ethical reasons but as well because these tests are little informative and so resource-intensive (time, infrastructure, personnel) that they cannot keep pace with testing demands. We therefore work on strategies that provide for chemical and water sample testing without the need of fish, with a prime focus being on fish cell lines as alternative models.

The star of our efforts to date is the RTgill-W1 cell line, which was established from the gill of a healthy rainbow trout (*Oncorhynchus mykiss*) in the early 1990s by Prof. Niels Bols [1]. Based on this cell line, together with a large team of enthusiastic supporters, we established, standardized and internationally validated an assay to predict fish acute toxicity, the so-called RTgill-W1 cell line assay [2-4]. We chose this cell line based on the assumption that it is the gill of fish that is mostly affected in acute exposure scenarios, leading to fish death. Viability of these gill cells is measured as proxy of fish death, showing an excellent correlation with a wide applicability domain for chemical properties, modes of toxic action and toxicities, with an exception being specific neurotoxic chemicals. The RTgill-W1 cell line assay has been adopted in 2019 by ISO (ISO standard 21115) and in 2021 by OECD (OECD TG249), marking the first *in vitro* ecotoxicology test guideline. We have meanwhile taken this assay into commercial testing services, receiving diverse requests for tests by chemical and consumer product industry, the aquaculture sector as well as environmental protection agencies, so much so that we are now establishing this assay under Good Laboratory Practice.

Indeed, not only are fish spared in this test, but it as well requires much lower test volumes and chemical amounts while being faster than conventional acute fish toxicity tests (according to OECD TG203) or even the zebrafish embryo toxicity test (zFET, OECD TG236), the latter of which is likewise considered an alternative to conventional tests with juvenile or adult fish. However, inasmuch as acceptance by regulatory agencies as standalone for a 1:1 replacement of the acute fish toxicity test seems currently unfeasible, we contribute to efforts to incorporate the RTgill-W1 and the zFET into Weight-of-Evidence approaches, such as Bayesian Networks, or into Integrated Approaches to Testing and Assessment. What is more, we are about to develop additional fish cell line-based modules to target prediction of non-acutely toxic chemical impacts to fish. Examples include predicting reduced fish growth, chemical bioaccumulation and intestinal inflammation. Future collaborations also foresee a focus on neuro- and reproductive toxicity.

Our presentation will focus on these most recent developments from a research point of view and then present examples of their practical use for chemical risk- and water quality assessment and for improving aquaculture and fish health.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The European ONTOX project: Safer chemicals using fewer animals

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The 3Rs concept, calling for replacement, reduction and refinement of animal experimentation, is receiving increasing attention around the world, and has found its way to legislation, in particular in the European Union. This is aligned by continuing high-level efforts of the European Commission to support development and implementation of 3Rs methods. In this respect, the European project called "ONTOX: ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment" was initiated in 2021 with the goal to provide a functional and sustainable solution for advancing human risk assessment of chemicals without the use of animals in line with the principles of 21st century toxicity testing and next generation risk assessment. ONTOX will deliver a generic strategy to create new approach methodologies (NAMs) in order to predict systemic repeated dose toxicity effects that, upon combination with tailored exposure assessment, will enable human risk assessment. For proof-of-concept purposes, focus is put on NAMs addressing adversities in the liver, kidneys and developing brain induced by a variety of chemicals. The NAMs each consist of a computational system based on artificial intelligence and are fed by biological, toxicological, chemical and kinetic data. Data are consecutively integrated in physiological maps, quantitative adverse outcome pathway networks and ontology frameworks. Supported by artificial intelligence, data gaps are identified and are filled by targeted *in vitro* and *in silico* testing. ONTOX is anticipated to have a deep and long-lasting impact at many levels, in particular by consolidating Europe's world-leading position regarding the development, exploitation, regulation and application of animal-free methods for human risk assessment of chemicals [1,2].

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Never waste a good crisis: Case study revealed faster regulatory approval of COVID-19 vaccine with fewer animal studies and more alternatives. Let's continue this promising road

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In a historical analysis we have revealed that regulatory requirements for animal studies in legislation were increased as a response to crises such as the Sulfanilamide elixer and Thalidomide disasters [1]. The Covid crisis in turn, led to a reversed situation, i.e., that fewer animal studies were requested. We have analyzed the case study of the fast-marketing emergency approval for the Pfizer/BioNTech mRNA vaccine Comirnaty through document analysis, stakeholder analysis and stakeholder interviews [2,3]. A much faster vaccine conditional marketing approval – in 10 months instead of the usual 10 years for a regular vaccine approval – was achieved by allowing fewer animal studies and more alternatives in the regulatory process [2]: a) only animal studies that were considered "essential" were required and more alternative methods were accepted, b) animal studies ran in parallel – instead of sequentially – with clinical trials, which meant that the results from animal studies were often not awaited before starting clinical trials, c) also historical results from earlier vaccines were accepted and d. batch releases were performed with alternative tests only, which was the result of the thorough characterization of the mRNA vaccine.

The COVID-19 pandemic crisis led to different routes for stakeholders like industry and regulators for vaccine development and – approval, as compared to "normal". Regulators used, e.g., rolling review procedures because of the emergency situation, which meant that already during the studies regulators would analyze the data instead of waiting for a full report encompassing all data of all studies at the end. In case the regulators would have awaited the study results from animal studies we could have found ourselves in a situation where we still wouldn't have had vaccines approved for the market.

Stakeholder interviews revealed that industry is clearly ready to work with alternatives more broadly, for scientific, economic, as well as ethical and societal reasons. Regulators appeared more hesitant to omit animal studies, associated with the perceived risk of backfiring in case of potential problems occurring after market approval. It will be necessary to examine how the confidence in alternatives can be improved, especially seen in the light of the fact that after animal testing and market approvals, also problems can arise which are currently not really seen as problematic but being part of the normal risks of the system.

Interdisciplinary transition and transformational governance research [4] will be important to understand how confidence in alternatives can be improved: this needs further development and validation of new alternative methodologies and processes (niche level), new legislative policies and guidelines (regime level) and societal/political research, e.g., on how risk assessment is performed and perceived (landscape level), and moreover, how all of these processes interact with each other. It is essential to have key stakeholders from at least industry and regulators communicate and collaborate from the start when developing/using new alternative procedures, in order to be able to implement those in practice fast, effectively and reliably. Interdisciplinary research is focused at bringing the different stakeholders together to facilitate these processes and make change happen.

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Submission declaration:
Conflicts of interest: The author declares that there is no conflict of interest.
Statement on ethics vote: Not applicable.

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Implementing new approach methodologies in EFSA's next generation risk assessment

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Since its creation in 2002, EFSA has made considerable investments into methodologies to collect, appraise and integrate scientific data and expertise to deliver state-of-the-science risk assessments in partnership with Member States, EU Agencies as well as international organizations and third countries. However, significant challenges still exist regarding the risk assessment (RA) of chemicals as a result of new methodologies reflecting increased understanding of how chemicals cause adversity as well as new types and mixtures of substances put in the market. EFSA also faces an increasing number of chemicals to be evaluated against a background of a large number of chemicals in food which still need to be assessed, as well as the increasing pressure from stakeholders for greater levels of information and reduced uncertainty.

EC policies such as the Green Deal and the Chemicals Strategy for Sustainability, and the new Regulation (EU) 2019/1381 amending Regulations (EC) No 178/2002 foster reflections on how to accelerate the pace of RA. A key action for EFSA is the development and implementation of new approach methodologies (NAMs) and tools to conduct RA without the need to conduct *in vivo* studies and which are capable of handling big data [1]. NAMs provide the opportunity to carry out chemical hazard assessments combining existing information with new information obtained from *in silico* and *in vitro* methodologies. The Next Generation Risk Assessment shall be hypothesis-driven and take advantage of new analytical techniques and high throughput/high content *in vitro* systems, based on human-relevant models. The integration of NAMs and Adverse Outcome Pathways (AOP) offers new opportunities for integrating mechanistic information, challenging the biological relevance of animal data as "gold standard" for risk assessment. This integration step is critical for the identification of susceptibility drivers and lead to a more informative risk characterization, improving consumer protection. A further integration of data from biomonitoring and post-market monitoring can widen the scope of exposure assessment bridging between external and internal exposures, resulting in a more precise risk assessment. The challenges for EFSA will be to i) apply these methodologies in a regulatory setting with sufficient confidence; ii) handle, curate, integrate and assess the large amounts of data obtained from these approaches (i.e., big data) and iii) balance them against the inherent yet familiar limitations of animal models in terms of sensitivity and human predictability.

EFSA has taken a number of initiatives since 2020 to tackle the above challenges in its chemical risk assessments. In particular, a call for the development of a NAMs roadmap on chemical RA for human health has been launched, and the recommendations provided in this roadmap [2] are being implemented in the form of case studies to demonstrate the advantages and limitations of NAMs in chemical RA. This presentation will provide an update on EFSA's scientific activities related to NAMs which are performed in close cooperation with EFSA's partners at the European and International level. Furthermore, it will address minimal and ideal requirements for using *in vitro* studies in regulatory risk assessments, guiding researchers on key elements of protocol design, scientific validation and reporting, that help ensuring the implementation and application of their results in regulatory assessments.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors. The views expressed in this publication are those of the authors and should not be interpreted as representing the official position of the European Food Safety Authority (EFSA). EFSA assumes no responsibility or liability for any errors or inaccuracies that may appear.
Statement on ethics vote: There is a positive ethics vote.

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Effect of CDE in RSV-infected human lung tissue *ex vivo* as alternative for animal-free testing of antiviral therapeutics

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The Respiratory Syncytial Virus (RSV) is a common cause of acute infectious diseases of the upper and lower respiratory tract. It is furthermore linked to the onset and exacerbation of chronic lung diseases, such as asthma. Still, the exact mechanisms are yet unknown as commonly used models resemble the related processes insufficiently. This limits the development of novel therapies. Up to now, there is no effective causal treatment for RSV infection and no vaccine has been approved for active immunization [1]. To investigate the immunological reaction to RSV infections, human based models, which reflect the local, immunological conditions in the airways are preferred for non-clinical studies. We established an *ex vivo* model of RSV infections on primary human lung tissue [2]. It can be used to investigate underlying pathophysiological mechanisms of RSV infections in a human setting and to test novel treatment options. Here, we tested the effect of a test substance (CDE) on RSV infections in human precision-cut lung slices (PCLS).

HEp-2 cells were infected with CDE pre-treated RSV for one hour. The number of infectious particles was quantified by Immuno-Plaque Assay after three days. Human PCLS were pre-treated with CDE for one hour and subsequently infected with RSV in the presence of CDE for two hours. Afterwards, CDE-treatment was either terminated, initiated or continued for another three days. Uninfected and untreated controls were included in each experiment. Tissue viability was measured by LDH assay and WST-1 assay. Secreted cytokines were quantified in the supernatants by multiplex ELISA.

CDE did not show any toxic effects on human lung tissue. It reduced the number of infectious particles in a dose-dependent manner *in vitro* (97.3% ± 2.0% reduction at 100 µg/mL; p 0.0108) and reduced the RSV-induced secretion of antiviral cytokines in PCLS (4-fold reduction of IFN-γ; p 0.0313; 4-fold reduction of IP-10; p 0.0313). Therapeutic treatment was more effective than pre-treatment, whereas a combination of both was most effective.

Our model resembles key biomarkers of RSV infections and provides a suitable platform to investigate certain characteristics of RSV infections in a human *ex vivo* setting. It can thus be used to characterize underlying pathophysiological mechanisms of RSV-infections and test novel treatment options *ex vivo* and reduce animal experiments. Overall, CDE did not affect cell viability in lung slices but reduced RSV load *in vitro* and prevented the RSV-induced antiviral immune response in human lung tissue *ex vivo*. CDE therefore creates a promising, novel, therapeutic option for RSV infections.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Microbiota dependent modulation of immune mediators in intestinal tissue slices *ex vivo*

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Several studies implicate a high relevance and impact of the gut microbiota on local as well as gut-distal immune responses. For instance, an altered intestinal microbiota composition with a prevalence of clostridial species has been described in asthmatic patients. Segmented filamentous bacteria (SFB) are members of the *Clostridiacae* family, which are known to induce Th17 cells in the gut and have been implicated to drive inflammation in experimental models, e.g., asthma. *Ex vivo* intestinal tissue slices (PCinS) are thin, viable tissue sections, which contain typical intestinal cell types including immune cells. The precision-cut organ slice technique allows to generate a high number of tissue slices from each region of the intestine, enabling parallel testing of multiple treatments and internal controls, while reducing the number of animals needed in line with the 3R principle. PCinS have been used for drug metabolism and toxicology studies [1]. However, the immune response in PCinS has not been characterized extensively, yet. In this study, we analyzed for the first time the local immune response in PCinS after restimulation *ex vivo* and compared relevant mediators in dependence of the microbiome composition of donor mice.

PCinS were prepared from the ileum of Taconic C57BL/6 mice. In a first set of experiments, general responsiveness of the tissue towards pro-inflammatory stimuli LPS and TNF-α was analyzed. Due to the known immunomodulatory role of SFB, we then specifically compared mice that either harbored SFB (Tac^+) or not (Tac^-). For restimulation of T-cells, PCinS were cultivated with Concanavalin A or anti-CD3/CD28 antibodies for 16 h. To determine the influence of the microbiome, co-housing of Tac^- with Tac^+ mice was performed. Tissue viability was assessed by LDH release assay and specific cytokine responses were measured via Multiplex (MSD) or ELISA. The microbiome composition of mice was analyzed in detail by whole-genome shotgun sequencing and effects on host tissue by RNA-sequencing.

PCinS remained viable over 16 h and were responsive to selected stimuli with secretion of multiple pro-inflammatory cytokines. Importantly, compared to PCinS from Tac^- mice, PCinS from Tac^+ mice displayed higher levels of specific cytokines such as IL-2, IL-17A, IL-23, IFN-γ and SAA1 (2-20-fold). PCinS from co-housed mice reacted similarly to PCinS from Tac^+ mice, demonstrating the role of the microbiome for the observed effects. In addition to SFB, we found that especially *L. johnsonii* was more prevalent in the ileum of Tac^+ and co-housed mice compared to Tac^- mice. RNA sequencing identified 393 genes that were induced dependent on the microbiome composition of mice, with *saa1* representing the top deregulated gene. Pathway analysis revealed enhanced T-cell signaling, interactions between lymphoid and non-lymphoid cells, synthesis of GPI-anchored proteins and metabolism of amino acids in co-housed vs. Tac^- mice. Of note, PD-1 signaling was found to be one of the main deregulated pathways depending on the microbiome composition of mice.

In summary, we here show for the first time that restimulation of immune cells, in particular T-cells, in PCinS *ex vivo* is feasible and dependent on the composition of the intestinal microbiome of mice. This offers new possibilities to study the influence of the microbiome on the immune system in a multicellular *ex vivo* system. Higher secretion of IL-23, SAA and IL-17A in PCinS from Tac^+ and co-housed mice fits the known role of SFB as potent stimulators of Th17 cells in the ileum. Of note, others have shown that soluble factors induced by SFB can drive the lung Th17 response during acute infection, depending on the presence of IL-1. SAA has been described to activate the NLRP3 inflammasome in mice and to promote neutrophilic airway inflammation in asthmatic patients. To study the effect of SAA as well as other gut-derived soluble factors on lung immune responses in more detail, PCinS can be combined with precision-cut lung slices (PCLS) in multi-organ-chips in future studies. Further, we currently transfer the knowledge gained from this study to human precision-cut intestinal slices, which enables us to investigate patient-specific immune responses *ex vivo*.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Phenomics and transcriptomics applied for key event identification resulting in an AOP network for developmental neurotoxicity

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New approach methodologies (NAMs) are non-animal-based methods, which can be used to provide information on chemical hazards. NAMs generate data on the toxicodynamics of a compound to support the proposed paradigm shift in toxicology – moving from the sole use of apical endpoints generated in animals towards a mechanistic understanding and more human-relevant approaches for regulatory applications. Information on toxicodynamics might arise from Adverse Outcome Pathways (AOP), which are structured organizations of causally related biological events leading to adverse effects and provide mechanistic information on the molecular initiating event (MIE), molecular, cellular, structural and functional key events (KE) and associated key event relationships (KER). Due to the manifold KEs that happen during brain development in a time- and brain region-specific manner, creation of AOPs for developmental neurotoxicity (DNT) that cover the DNT toxicological space will be a continuous effort that is far from being completed at the moment. To date, seven DNT-AOPs are endorsed by the OECD, eleven were submitted to the AOP Wiki and some were published in the scientific literature. Another challenge for creating DNT-AOPs is based on the fact, that most of the assays used for DNT represent several KEs, but do not directly measure MIEs.

We have developed 3D neurosphere *in vitro* test methods (NPC1-6) based on human primary neural stem/progenitor cells (NPCs), which represent distinct neurodevelopmental KEs, including NPC proliferation, migration and differentiation into neural effector cells (radial glia, astrocytes, neurons and oligodendrocytes), as well as thyroid hormone (TH)-dependent oligodendrocyte maturation. Using these neurosphere test methods, we evaluated the endophenotypic adverse effects of several compound classes on these neurodevelopmental processes. Additionally, we performed microarray analyses for gaining deeper mechanistic understanding and identifying MIEs. Finally, data were placed in an AOP-context.

All compounds tested in this study caused DNT endophenotypes, i.e., disturbed radial glia migration, impaired oligodendrocyte differentiation as well as oligodendrocyte maturation. Transcriptome analyses complemented these cellular phenotypic observations with molecular data. Here, we identified three independent MoAs interfering with oligodendrocyte development: (i) Thyroid Hormone (TH) disruption via receptor binding, (ii) reactive oxygen species accumulation along with oxidative stress and (iii) disturbance of cholesterol homeostasis. Furthermore, we confirmed a migration endophenotype caused by protein interaction, i.e., compound binding to the extracellular matrix protein laminin thereby leading to a disturbance in radial glia adhesion. Finally, all identified MIEs and KEs, in combination with literature-based KEs were combined in a novel putative AOP network which refers to adverse outcomes regarding cognitive, attentional, behavioral and socialization deficits, clinical signs of neurodevelopmental disorders.

This study demonstrates the power of combining endophenotypic with transcriptomic analyses for better understanding MoA and building AOPs for DNT, a promising approach for future risk assessment procedures without the use of animals.

Submission declaration:
Conflicts of interest: There is no conflict of interest.
Statement on ethics vote: hNPCs were purchased from Lonza Verviers SPRL, Belgium and work was approved by the ethics committee of the Heinrich-Heine University Düsseldorf.

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Landscape new approach methodologies (NAMs) for safety assessment of chemical substances

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In the Landscape NAMs, RIVM provides a current overview of who is involved, which initiatives are undertaken, and which trends can be observed in the implementation of NAMs for the safety assessment of chemical substances [1]. Gaining insight in the types and roles of organizations involved is an essential first step to facilitate the implementation of NAMs.

Chemical substances need to be safe for human health and the environment. Various EU-regulations are in place to ensure the safety of chemical substances. These regulatory frameworks require toxicological information to assess safety. The information needed for a safety assessment is obtained from a number of different tests, including animal studies. In the last decades, increasing effort is made towards the implementation of 3Rs, to replace reduce and refine these animal studies. A trend in the context of safety assessment is the use of the term NAMs, to represent *in vitro*, *in chemico* and *in silico* approaches as replacement methods for animal studies.

The basis of the Landscape NAMs is the implementation curve. This curve illustrates the phases that a NAM typically follows in order to be implemented in regulatory frameworks. In every phase of this curve there are international organizations and advisory bodies involved. They can influence and stimulate the validation, acceptation, and implementation of NAMs. The phases from validation to uptake in legislation and regulation mainly take place in an international setting where various (international) organizations and advisory bodies are involved in consultation and decision-making about test methods. Cooperation between the stakeholders in and between the phases is essential for efficient implementation of NAMs.

While the Landscape NAMs presents the Dutch perspective, it provides a framework for other countries to get more insight in the representation of their organizations in (inter)national committees, networks and projects in order to facilitate the implementation of NAMs for chemical safety assessment.

Reference

[1] https://www.rivm.nl/documenten/landscape-new-approach-methodologies-namssafety-assessment-chemical-substances

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

145

VHP4Safety: Assessing the safety of chemicals and pharmaceuticals without using laboratory animals

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VHP4Safety will develop the world's first Virtual Human Platform to determine the safety of chemicals and pharmaceuticals for human health based solely on human biology [1]. By integrating innovations in data science, human tissue culture models and transition management, VHP will spearhead the transition to animal-free safety assessment.

The mission of the VHP4Safety is to improve the prediction of the potential harmful effects of chemicals and pharmaceuticals based on a holistic, interdisciplinary definition of human health and accelerating the transition from animal-based testing to innovative safety assessment. The VHP4Safety integrates data on human physiology, chemical characteristics and perturbations of biological pathways in an inclusive and integrated manner that incorporates: 1) human-relevant scenarios to discriminate vulnerable groups (such as disease state, life course exposure, gender and age); 2) chemicals from different sectors (pharma, consumer products and chemical industry); and 3) different regulatory and stakeholder needs.

The VHP4Safety platform will be developed within three interacting research lines that involve building the platform, feeding the platform with newly generated data, and implementing the platform to ensure stakeholder acceptance, governance and sustainability.

VHP4Safety addresses the emerging societal challenge of the transition to animal-free safety assessment, by integrating various scientific disciplines in the consortium and working with all stakeholders towards implementation and societal acceptance of an approach to chemical safety assessment that is based on human data rather than animal data.

The VHP4Safety consortium consists of leading scientific groups from Dutch universities, university medical centers, public health institutes and applied research organizations, with expertise spanning the technological, biological, chemical, medical as well as the social sciences. Co-funders and cooperation partners ensure the active involvement of diverse academic, regulatory, industrial and societal partners to the project throughout the entire safety assessment knowledge chain. As such, the VHP4Safety consortium unites necessary and complementary expertise to build, test and evaluate the platform.

VHP4Safety is a research project funded by the Dutch Research Council (NWO) program entitled the "Dutch Research Agenda: Research on Routes by Consortia (NWA-ORC)." With a budget of over 10 million Euros, the project starts on June 1, 2021 and will last for the duration of 5 years.

Reference

[1] https://vhp4safety.nl/

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

146

Neutralizing effects of different antibodies on *Clostridioides difficile* toxins TcdA and TcdB in a translational approach

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Given the high prevalence of intestinal disease in humans and animals, there is a strong need for clinically relevant models recapitulating gastrointestinal systems ideally replacing *in vivo* models in accordance with the principles of the 3R. Intestinal organoids allow the study of interactions between the gut microbiota and gut epithelium. Hence, exploring ways to mitigate disease-provoking effects of pathogens and/or boost health-promoting effects of natural commensals is possible using intestinal organoids. We have previously established canine intestinal organoid models such as jejunal and colonic organoids, which can function as epithelial models that faithfully mimic respective intestinal sections [1]. These models can help to bridge the gap between *in vitro* screening and *in vivo* assessment of possible therapeutic drugs.

Here, we report on the use of jejunal and colonic organoids to analyze the neutralizing effects of recombinant versus natural antibodies on *Clostridioides difficile* toxins *TcdA and TcdB.* To this end, organoid-derived monolayers were compared to three-dimensional organoids. In addition to the conventional "basal-out" organoids, we generated so-called "apical-out" organoids to more easily access the apical cell surface for toxin binding. Recombinant antibodies comprised the IgG antibody bezlotoxumab, which is in clinical use for the treatment of *C. difficile* infections [2,3] and a recombinant sIgA established by us [4]. The natural antibodies are sIgA antibodies purified from pooled goat whey.

Analyses of the respective binding affinities of these antibodies to both toxins were carried out by ELISA. Subsequently, sulforhodamine B cytotoxicity and FITC-dextran barrier integrity assays were used to establish the antibodies' respective toxin neutralization profile in the organoid model. We show that recombinant sIgA proved equally effective in neutralizing the cytotoxic effect of TcdB on canine intestinal organoids and organoid-derived monolayers as IgG, whilst goat sIgA failed to sufficiently neutralize TcdB in these *in vitro* systems.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: Tissue sampling was approved by the institutional ethics committee in accordance with Good Scientific Practice guidelines and Austrian legislation. Based on the guidelines of the institutional ethics committee, use of tissue material collected during therapeutic excision or post mortem is included in the University's "owner's consent for treatment", which was signed by all patient owners.

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Development, efficacy and safety of a novel therapeutic protocol against an experimental model of human malignant melanoma

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Malignant melanoma is one of the most aggressive types of human cancer, with a high fatality rate, due to its resistance to current therapies. On the other hand, the extensive deregulation of normal epigenetic marks is associated with melanoma onset and progression. In this context, given the well-established association between epigenetic modifications and alterations in gene expression, together with the reversible nature of such modifications, it becomes apparent that they have attracted scientific interest as potential therapeutic targets [1]. On the other hand, isothiocyanates [(ITCs); the bioactive compounds of cruciferous vegetables derived from the hydrolysis of glucosinolates (GLs)], have been previously reported to possess a significant anticancer activity against various types of cancers, including melanoma. Specifically, their anti-melanoma effect has been suggested to be mediated through induction of apoptotic cell death and cell cycle growth arrest as well as inhibition of the metastatic and migratory potential, among others. Only recently, their role as potent epigenetic regulators has emerged in the context of being capable of reversing the aberrant epigenetic landscape and ultimately leading to melanoma cell death [2-4]. In the present study, we aimed to characterize the therapeutic effect of three epigenetic drugs namely (i) Zebularine (a DNA methyltransferase inhibitor), (ii) Entinostat (a histone deacetylase inhibitor and (iii) Tazemetostat (a specific inhibitor of EZH2 histone methyltransferase) alone or in combination with different ITCs [Sulforaphane (SFN); Iberin (IBN); Phenethyl (PEITC) and Benzyl (BITC) ITCs] by utilizing an *in vitro* model of human malignant melanoma (A375 and Colo-679) and non-tumorigenic immortalized keratinocyte (HaCaT) cells. The latter cell line was utilized as a control, non-malignant one (which predominantly exists in the epidermis and thus surrounds a malignant melanocyte) allowing us to determine drug safety by means of any potentially observed cytotoxicity. Briefly, cells were exposed to (i) either epigenetic drugs as single treatments or (ii) various combinatorial schemes involving different classes of ITCs in order to determine their cytotoxic profile and characterize their overall potential. Our results revealed, for the first time, that exposure to all tested epigenetic drugs significantly reduced cell viability levels, in a dose- and time-dependent way, by activation of apoptosis in human malignant melanoma cells whereas non-malignant keratinocyte cells remained unaffected. In addition, combinatorial treatments, with ITCs, resulted in further reduction of cell viability levels an effect that was accompanied by higher apoptotic rates, as evidenced by increased levels of caspase-3 activity. Overall, we have developed an experimental therapeutic protocol based on novel combinatorial conditions where we have enhanced the anticancer action of clinically relevant epigenetic drugs, at minimal concentrations, thereby ensuring increased therapeutic potency, while we maintain safety, against human malignant melanoma cells.

This project has received funding from a grant provided by the Cyprus Institute of Neurology and Genetics (Telethon Cyprus), Nicosia, Cyprus

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Submission declaration:
Conflicts of interest:The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote:There is a positive ethics vote.

148

Implementing microfluidic systems into the 2D and 3D cultures: Challenges and benefits of the flow

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Over the last several decades a number of research teams developed advanced *in vitro* cell-based systems that try to better mimic the physiological conditions of animal and human cells and organs *in vivo*. This has led to the evolution from planar cell culture (2D) to the cell culture on 3D scaffolds, and the incorporation of cell scaffolds into microfluidic devices [1].

In our work, oriented on the development and validation of methods applicable for the biocompatibility testing of medical devices, we tried to compare the effect of static and dynamic conditions using mouse fibroblasts (a 2D model recommended by the ISO 10993-5) and 3D reconstructed human tissue model mimicking the human cornea and epidermis. Models were cultures within a fluid-dynamic bioreactor (MIVO®) device able to mimic the capillary flow dynamics.

Early cell adhesion, metabolic activity and proliferation were investigated by culturing 3T3 cells in the static and dynamic conditions with several modifications of flow rate for 7 days in the respective media with and without serum. A similar experiment was conducted with 3D tissue models mimicking human cornea and epidermis.

The pilot study conducted with 3T3 mouse fibroblasts showed differences in the cell shape and orientation towards the flow. The metabolic activity and proliferation seemed to be unaffected in the slowest flows. In the case of the 3D models, the media circulation led to slight metabolic and morphological changes. These findings are useful considering their application in tissue engineering in order to mimic as close as possible the physiological conditions for instance in the cardiovascular system.

This work was supported by the APVV project APVV-19-0591 and VEGA Project. Vega č. 2/0153/20

Reference

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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A3RC – Austrian 3R Center update

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The RepRefRed Society, Austrian's 3R association, was assigned by the Federal Ministry for Education, Science and Research in 2019 to establish the Austrian 3R Center (A3RC). Three years after starting the project the Austrian 3R Center launched its first focus event about "Unused surplus animals from laboratory animal breeding" in June 2022 with 150 registrations. During the last years the RepRefRed Society has been putting much effort in networking and improving its online presence in order to pave the way for the Austrian 3R Center.

The purpose of the A3RC is to create a national point of contact as well as a national knowledge platform for the 3Rs and related topics. It aims on connecting researchers from the academics and the industry to support the 3Rs.The goal of the A3RC as well as the RepRefRed Society is to provide balanced information about all the 3Rs and not to focus on a specific R. Beside the general knowledge transfer the A3RC yearly coordinates its focus topics in cooperation with its funding source, the Federal Ministry for Education, Science and Research.

In cooperation with the RepRefRed Society the Austrian 3R Center should strengthen Austria's position in the European 3R landscape. Both institutions are members in the "Network of European 3R Centres – the EU3Rnet".

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Identifying endocrine disruptors via a HTS platform based on the Sandell-Kolthoff reaction

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Toxicological *in vitro* studies on adverse effects of chemicals or natural compounds disrupting the thyroid hormone (TH) system usually focus on histo-morphological changes of the thyroid gland as readout, as well as altered concentrations of the hormonal parameters T4, T3 and TSH in the blood. However, effects on such intermediate key events need further specification regarding their mode- of-action (MoA) at the tissue- and cell-specific level, defining the molecular mechanism mediating the endocrine (adverse) effect as required for the definition of an Endocrine Disruptor. Such local THSD targets are transmembrane TH transporters (e.g., MCT8), dehalogenase 1 (DEHAL1) and intracellular deiodinases (DIO1, 2, 3) [1]. These proteins are key regulators for the control of local TH action. To address these local targets of the TH system, we established MIE-specific High-Throughput-Screening (HTS) assays based on the versatile and robust Sandell-Kolthoff reaction. 60,000 compounds were already tested for their interferences with DIO2, nearly 500 compounds were tested for DEHAL1. An MCT8 HTS Assay has been established now and an initial screening is ongoing.

The High-Throughput-Screening (HTS) assay on DIO2 inhibition uses recombinantly expressed human DIO2 enzyme and large compound libraries comprising small molecules, environmental toxins such as pesticides and FDA-approved drugs, along with other chemicals, were tested at a concentration of 10 µM. 977 compounds showed a DIO2 inhibition by more than 25% and 106 compounds an inhibition by more than 50%. 352 compounds were selected for analyzing IC50 values and 24 potent DIO2 Inhibitors were identified. Interestingly, FDA-approved drugs and supplements, as well as pesticides were among the identified DIO2 inhibitors. Furthermore, a cellular MCT8 HTS assay was set up and an initial screen is already ongoing using MDCK1 cells overexpressing human MCT8. For the DEHAL1 screening nearly 500 compounds were tested in duplicates using recombinantly expressed human DEHAL1. Twelve compounds with a DEHAL1 inhibition of more than 30% were chosen for IC50 analyses and two compounds were identified as potent DEHAL1 inhibitors with an IC50 value lower than 25 µM.

The presented work as essential part of the EU-funded ATHENA- project demonstrates the successful setup and application of a HTS-screening platform, qualified to identify reference compounds, and to detect and initially characterize endocrine disruptors, affecting DIO, DEHAL and MCT8 activities. The generated data pools provide the basis to select Quantitative Structure Activity Relationship training sets to develop predictive *in silico* tools for preselection and toxicological assessment. Furthermore, this setup opens the perspective to identify highly potent drug candidates with a potential role in treatment of endocrine and endocrine-related diseases. The established HTS assay protocol also provides the basis for testing of large chemical libraries against other deiodinating enzymes modulating TH availability, e.g., DIO1, as well as other thyroid hormone transporters.

This project is embedded within the EU H2020 funded ATHENA consortium, aiming i) to develop *in vitro* screening assays for EDCs affecting components of the THS especially during early fetal, embryonal and perinatal development, when maternal (placental) TH supply is crucial for brain development, ii) to identify potential reference compounds specifically targeting individual components of the THS, and iii) to challenge these *in vitro* assays for their translational utility in regulatory testing of EDCs [2].

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Animal-free *in vitro* – Replacement of animal-derived products within the OECD TG 487 and 455 – Phase 1 report

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Introduction: The next step to advance *in vitro* toxicology is the replacement of animal-derived components like fetal bovine serum (FBS), rat liver homogenates (S9) and plasma proteins such as the albumins in effect-based methods. These animal-derived products must be substituted as they are a source of poor bioassay reproducibility [1,2]. In this study, we report the phase 1 results of the NC3R CRACK IT Challenge 36: animal-free *in vitro.* This challenge aims to replace animal-derived products in the OECD test guidelines 487 (mammalian cell micronucleus test) and 455 (detection of estrogen receptor agonists/antagonists) with animal-free and preferably chemically defined media for growth and assay medium and, if necessary, metabolic activation with animal-free alternatives.

Material and methods: For this study, adherent A549 (OECD TG 487) and ER-CALUX® (OECD TG 455) cell lines were chosen, and their growth was monitored using manual or automated microscopy and confluency determination. In a first step, 30 to 37 chemically defined media (CDM) combinations and four chemically defined FBS replacements have been evaluated in both TGs in an inside adaptation or stepwise reduction of serum content approach. Additionally, for the OECD TG 455, candidate media and supplements were investigated for interference with the assay readout. In a second step, we investigated the replacement of S9 with a biotechnologically produced cocktail of metabolic enzymes (ewoS9R) for the OECD 487. Finally, in a third step, we investigated the implementation of metabolic activation into the OECD TG 455 via S9 and ewoS9R. For comparison, ewoS9R and rat liver S9 amounts were calculated using the bioassay's final protein content of 0.1 mg protein/mL rather than the imprecise percentage (v./v.) nomenclature [3]. As a reference compound for both OECD TGs, benzo[a]pyrene (B[a]P) was chosen, as it causes after metabolic activation both genotoxic effects via micronucleus formation and endocrine activity. BaP was evaluated in a concentration range of up to 10 µM.

Results and discussion: The stable adaptation of two relevant cell lines to chemically defined media (CDM) was achieved. The A549 cell line could be cultivated in 95% (v./v.) X-VIVO15 (Lonza) + 5% (v./v.) Panexin CD (PAN Biotech). The doubling time for the A549 cell line increased from 24 h in the FBS-containing medium to 46-52 h in CDM. The performance of the micronucleus assay was impeded as the cells could not be distributed homogenously, and the formation of cell aggregates made the detection of micronuclei impossible. Concerning the OECD TG 455, the ER-CALUX® cells could be cultivated in a CDM consisting of X-VIVO 10 (Lonza) as the growth medium. The adaptation and conduction of the OECD TG 455 variant with CDM could be reproduced within two laboratories (GU and BDS). In CDM, the doubling time increased from 24 h in the FBS-containing medium to 40-48 h. Due to an intrinsic endocrine activity of X-VIVO 10, a mixture of 50% (v/v) DMEM-F12 and 50% (v./v.) X-VIVO 10 was determined as the assay medium. In CDM, the OECD 455 assay showed a reduced response intensity of the cells. The signal was approx. < 5% of the FBS-containing medium variant. The sensitivity to the reference compound E2 was also reduced as the EC50-values in CDM were at GU-lab: 22-78 pM and BDS-lab: 24-30 pM compared to the FBS-containing medium with an EC50 of 4-8 pM (GU/BDS). This result could be explained by the lower protein content of the CDM and thus reduced compound availability.

The biotechnological metabolization system ewoS9R could be implemented within both TGs. Concerning the OECD TG 487, ewoS9R could only be implemented in the FBS-containing variant. Here, B[a]P+S9 showed genotoxic effects at 0.625, 2.5, and 5 µM B[a]P, and with B[a]P+ewoS9R at 1.25, 2.5, 5 and 10 µM B[a]P.

Within the OECD TG 455, ewoS9R could be implemented into both variants (CDM and FBS-containing). B[a]P alone showed a low dose-independent endocrine activity in all OECD 455 variants. After metabolization via S9 or ewoS9R of B[a]P, the estrogenic activity increased with B[a]P-concentration in both FBS-containing medium and CDM. To summarize, the culture of OECD-relevant cell lines can be achieved in an animal-free variant. However, further research is needed to improve the effect-based methods' conduction and performance.

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Submission declaration:
Conflicts of interest: The corresponding author declares that Andreas Schiwy, Beat Thalmann and Henner Hollert are co-founders of EWOMIS GmbH, a company aiming to commercialise biotechnological metabolisation systems.
Statement on ethics vote: No ethics vote required.

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Development of fish liver PLHC-1 spheroids and their applicability to investigate the toxicity of plastic additives

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Fish liver cell lines are valuable tools to understand the toxicity of chemicals in aquatic vertebrates. Conventional 2D cell cultures grown in monolayers are well established but have some limitations since they fail to emulate toxic gradients and cellular functions as in *in-vivo* conditions. To overcome these challenges, 3D spheroid-based *in-vitro* platforms have been successfully implemented in biomedical research. However, the development of 3D-cultures from piscine species and their application to aquatic toxicity studies is still in its infancy. This work focuses on the development of *Poeciliopsis lucida* (PLHC-1) spheroids as a testing platform to better simulate the microstructure and environment of fish liver cells, and to assess the toxicity of a mixture of plastic additives.

PLHC-1 cells spheroids were formed in ultra-low attachment plates, and parameters such as spheroid size, seeding density and cell viability were monitored over time. Spheroids of 2 to 8 days and 150-250 µm size showed excellent viability and were further selected for toxicity studies. The lipidomic analysis of day-8 spheroids allowed the identification of about 170 individual lipids, including phosphatidylcholines (PCs), phosphatidyl-ethanolamines (PEs), ether link PEs/PCs, sphingomyelins (SMs) and cholesterol esters. Spheroids showed a completely different lipidic profile than 2D-cells, characterized by a relative enrichment of highly unsaturated PCs, sphingosines, SMs and CEs, when compared to cells growing in monolayers.

Both, 2D-cell cultures and spheroids were used as *in vitro* models to investigate the toxicity of a mixture of 10 common plastic additives, including bisphenol A and derivatives, phthalates, alkylphenols, triclosan, and tritolylphosphate. Cell monolayers and spheroids were exposed to different concentrations of the mixture tested at an equimolar concentration (0.1 to 5 µM). A significant reduction in cell viability (80%) and a 2-fold increase in the generation of reactive oxygen species (ROS) were observed in PLHC-1 monolayers (0.5 to 1.5 µM), while spheroids showed higher resistance to mixture toxicity. However, a high number of lipids were up-/down-regulated in spheroids after exposure to the mixture of plastic additives, suggestive of much higher sensitivity to exposure to the plastic additives in terms of modulation of lipid metabolism.

Overall, this work evidences the suitability of PLHC-1 spheroids as a testing platform. The lipid profile of 3D-spheroids is indicative of a liver-like phenotype, which represent an important step towards the application of more realistic *in-vitro* methods in aquatic toxicity studies.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Retrofitting *in vitro* test systems for high throughput screening of thyroid disrupting chemicals with external biotransformation systems – A critical review and tiered *in vitro/in silico* approach

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Whilst many chemicals may be metabolized, detoxified and excreted *in vivo*, it is of special (eco)toxicological interest that some reveal their toxic potential only after bioactivation. The latter is of regulatory concern internationally, particularly for endocrine active chemicals [1-3]. High throughput screening (HTS) approaches that screen for endocrine activity do not include xenobiotic metabolism. Most *in vitro* test systems do not preserve the metabolic activity of their parent tissue of origin. Therefore, combining *in vitro* test systems with metabolic capacities is a promising solution to this concern. However, current approaches (e.g., primary cultures, spheroids) hamper HTS applications due to costs and technological practicality.

Nevertheless, cellular test systems augmented with external metabolism capacities (liver S9 fractions and alternatives) appear to be adjustable to HTS scenarios, as shown for estrogen and androgen receptor (ant-) agonistic effects, if parameters are appropriately adjusted [4]. As part of the EU H2020 ERGO project [5], a battery of *in vitro* bioassays for assessing thyroid hormone (TH) disruption is being developed to populate future TH cross-species AOP networks. Here, we report on the steps towards introducing the biotransformation aspect into HTS assessment of the TH disrupting potential of chemicals.

In tier 1 of the project, an in-depth critical assessment of the literature regarding the utilization, characterization, and reporting standards of externally added biotransformation systems (BTS; S9 and microsomal liver fractions) has been conducted (n = 219). The intention was to derive optimal concentration and activity parameters during a 24 h co-metabolization exposure. Neither statistical correlations nor methodological consistencies could be extracted for BTS and respective cofactors from the meta-analysis of spatio-temporal, concentration, and activity patterns. As encountered in the literature, the shortcomings of BTS reporting standards hinder the replication and interpretation of generated data, leading to inconsistencies when the data is applied *in silico*. A critical review article, in accordance with PRISMA and EFSA guidelines, is currently in preparation and will outline reporting recommendations ensuring reproducibility, comparability, and parameter optimization for BTS, such as reporting on BTS protein concentration and phase 1 and 2 enzymatic activities.

In tier 2, two HepG2-based reporter cell systems (TH metabolism, TH signaling), an assay for iodide uptake (critical for TH synthesis), and a TH transporter (TTR) assay were assessed regarding their compatibility with BTS in parallel to chemical exposure. As deducted from our analysis in tier 1, the parameters for potential cytotoxicity, cofactor concentrations, and phase 1 and 2 enzymatic activity (CYP1A, CYP3A, GST, UGT, SULT) were assessed and optimized. We report on the functionality of the TH-BTS *in vitro* battery within the co-metabolization/exposure scenario as an HTS approach. Further, we outline how BTS interferes with assay readouts and describe preventive strategies. Primarily, rat-derived BTS have been used throughout the experiments but will be replaced once cell culture-derived BTS (e.g., ewoS9) have reached complete phase 1 and 2 metabolic maturity. Nevertheless, lessons learned from tiers 1 and 2 will be critical for future characterization and utilization of animal-free BTS to avoid historical omissions.

In tier 3, *in silico* biotransformation models (Biotransformer, QSAR toolbox, CTS) are employed to derive consensus metabolites, which are assessed for a potential reduction or increase in toxicity via TR a/b and TTR-inhibition QSARs. The *in-silico* data will be qualitatively compared with the *in vitro* results.

The HTS testing scenario complies with devised OECD short and midterm strategies for *in vitro* endocrine activity metabolism assessment [2,3]. Indeed, S9 liver fractions are animal-derived components that should be phased out as soon as alternatives are available. However, liver S9 fractions currently serve as a consistent regulatory-accepted biotransformation tool within *in vitro* toxicology, as seen in genotoxicity testing, until biotechnological alternatives have matured.

This project has received funding from the EU H2020 project ERGO (No.825753).

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Submission declaration:
Conflicts of interest: The authors declare that Andreas Schiwy is a co-founder of EWOMIS GmbH, a company aiming to commercialize biotechnological metabolization systems.
Statement on ethics vote: No ethics vote needed.

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Single cell sequencing to study IL-1β induced changes in chondrocyte pellet cultures

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Today's greatest challenge in biomedical research on osteoarthritis (OA) is the selection and reliability of biological models to study underlying disease mechanism. To reduce the usage of animal models, *in vitro* methodologies based on human material have gained increasingly attention during the last decade. Chondrocyte pellet cultures present one of the most often utilized tools to recapitulate changes in cellular phenotypes and extra cellular matrix (ECM). Although standard histological, biochemical, and molecular read-out parameters indicate promising overlaps between *in vitro* pellet cultures and *in vivo* cartilage features, precise in-depth analysis on the cell heterogeneity on transcriptomic level with respect to pathophysiological changes induced by, e.g., interleukin-1β (IL-1β) are missing. Recent studies on cartilage samples from OA patients using single cell RNA sequencing (scRNA-seq) techniques suggest that cell heterogeneity increases and dramatically shifts during OA revealing distinct cellular states within normal and diseased cartilage. Here we present preliminary data on the optimization of the methodological pipeline to delineate cell heterogeneity in human chondrocyte pellet cultures using scRNA-seq including technical details on pellet culture conditions, pre-sorting approaches to enrich viable cells, and considerations to preserve the transcriptome from dissociation-induced alterations.

After expansion, 2.5 x 10^5 human primary chondrocytes were resuspended in chondrogenic medium (StemMACS™ ChondroDiff Medium). Cells were pelleted by centrifugation (300 x g, 8 min) in 2 mL conical tubes. Pellets were kept in static culture for up to 21 days in closed tubes in a CO_2 incubator. First, pellet maturation was monitored by analyzing ECM formation (Alcian blue staining; day 7 and 21) and mRNA expression of *COL2A1, ACAN* and *COL1A1* at day 7, 14 and 21 (n = 3). Next, different concentrations of recombinant human IL-1β (0.01, 0.1, 1.0 or 10 ng/mL, 2 x 108 IU/mg) were supplemented at day 1 to evaluate effects on matrix maturation and cell phenotypes. Alcian blue staining and mRNA expression of *COL2A1, SOX9, HSPB1* and *IL6* were performed at day 21. To determine a suitable method to dissociate the cells from the pellets, mechanical and enzymatic digestion with 1 U/mL (Wünsch) collagenase D for 2 h or 4 h at 37°C were compared based on resulting cell viability using 7- and 21-day old pellets. In addition, the effect of supplementing the transcription inhibitor Actinomycin-D (Act-D; 2 µg/mL) [1] was analyzed using flow cytometry (cell viability) and qPCR (*HSPB1, COL2A1, CTGF, SOX9, CYR61*).

Analysis of chondrocyte pellet maturation over 21 days revealed a loose ECM structure with homogenously distributed cells at day 7 and a dense ECM structure with elongated cells in the superficial zone and rounded cells in the pellet center at day 21. On gene expression level, *COL2A1* was increasingly expressed over time accompanied by a reduced expression of *COL1A1* and *ACAN*. Supplementation of IL-1β resulted in ECM changes as investigated by Alcian blue staining at day 21. Therefore, less Alcian blue positive areas were observed in pellets treated with 1 ng/mL IL-1β and complete loss of Alcian blue staining was found in pellets treated with 10 ng/mL IL-1β. Alcian blue staining of pellet cultures treated with less than 1 ng/mL IL-1β revealed rather phenotypical than ECM-related changes. *COL1A1, SOX9* and *HSPB1* expression was only slightly altered in the different treatment groups at day 21, while *COL2A1* expression decreased by adding 1 and 10 ng/mL IL-1β. This is accompanied by an increased expression of ECM degrading and cell morphogenic enzymes. Cell dissociation experiments suggested that mechanical isolation has a negative impact on cell viability and an overall lower cell output. For collagenase digestion, 2 h incubation time yielded similar cell numbers as 4 h incubation while exhibiting higher viability (day 21). Adding ActD to the collagenase digestion resulted in significant reduction of cell viability. However, gene expression analyses revealed the intended effect of transcription inhibition by strong reduction of the fast-acting genes *CTGF, CYR61* and *SOX9*, while *COL2A1* and *HSPB1* expression remained unaffected. Based on our preliminary results, chondrocyte pellets will be cultured for 21 days for the following scRNA-seq experiments comparing three conditions i) control without IL-1 β, ii) supplementation of either 0.1 ng/mL IL-1β or iii) 10 ng/mL IL-1β to recapitulate different stages of cartilage degeneration. Despite the apparent simplicity of pellet cultures – optimized, reproducible conditions and protocols are indispensable to precisely recapitulate presumed *in vivo* conditions to facilitate the translational value.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Metabolisation system to improve the forecast power of the fish embryo acute toxicity test (FET)

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The Fish Embryo Acute Toxicity (FET) Test, according to OECD TG 236, is an early-life-stage test to evaluate lethal and sublethal effects of chemicals/chemical mixtures on the organismic level. It is conducted with zebrafish (*Danio rerio*) larvae during the first days of development post fertilization [1]. It was developed and standardized as a substitute for the acute fish test according to OECD TG 203, in which effects of the test substance/mixture on adult fish are assessed. The use of larvae is considered as a replacement of the more sentient adult animals according to the 3 R's principle (reduction, refinement and replacement of animal experiments) [2]. However, *Danio rerio* larvae have only limited metabolic capacity during the development until 96 hours post fertilization compared to adult fish [3,4]. This can result in an under- or overestimation of the toxicity of environmental samples or chemical substances. Therefore, the FET is poorly established/accepted in the regulatory context as an alternative to the acute fish test.

This project is intended to investigate whether the FET could be further improved by a preceding metabolism of the sample. The goal is to strengthen its forecast power and improve its acceptance for regulatory risk assessment. To achieve this goal a pre-metabolization procedure will be optimized and implemented into the OECD TG 236. For pre-metabolization, a biotechnological, animal component-free S9 (ewoS9R) and a conventional S9-fraction (rat liver homogenate) are evaluated and compared. Both metabolization systems are used to enable the detection of the metabolite's effects in the FET. The effects observed during the test using pre-metabolized substances will be compared to the effects resulting from the non-metabolized parent compounds.

For this study, five model substances (Benzo[a]pyrene, Bisphenol A, Chlorpyrifos, TDCPP, Carbamazepine) are applied. For each of these compounds, bioactivation is expected to increase their toxicity. It is assumed that both testing approaches result in different concentration-response curves, where (possibly different) (sub-)lethal effects may occur at lower concentrations with pre-metabolized substances compared to the approach using parent compounds. Through biotransformation, stronger or weaker effects could then be monitored in the FET, which may better reflect the results from the acute fish test with adult fish with full metabolic capacity. The final goal is to develop an applicable method to make the FET results more comparable to the acute fish toxicity data and therefore even more relevant for risk assessment.

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Submission declaration:
Conflicts of interest: The corresponding author declares that Andreas Schiwy and Henner Hollert are co-founders of EWOMIS GmbH, a company aimed to commercialize biotechnological metabolization systems.
Statement on ethics vote: No ethics vote required.

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ReThink3R 2.0 Summer School – Educating the next generation of scientists

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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 3Rs topic and completely rethink the used methodology with respect to new technologies in a satisfying manner. In addition, training early career investigators in 21st century skills such as critical thinking, collaboration, creativity, and empathy is critical to empower the next generation of scientists. In order to tackle these challenges, we developed an interactive workshop concept (ReThink3R) using Design Thinking methods to train early career investigators to generate innovative ideas and solutions towards the implementation of the 3Rs principle in research. To make this concept accessible to a larger number of early career investigators and booster the implementation of bioinformatic tools, we are currently organizing a 4-day Summer School to be held on August 23-26 in Berlin, Germany with 42 PhD students and postdocs from Germany, Europe, India, and the US. We want to bring together international early career investigators from various backgrounds in computer science, biomedicine, or laboratory animal science to promote interdisciplinary exchange and lay the foundation for potential subsequent cooperation. During the ReThink3R 2.0 Summer School early career scientists work together on innovative solutions for the implementation of the 3Rs in science with a specific focus on biomedical research and bioinformatics by exchanging knowledge, networking, and joining forces in a discipline-spanning manner during the Design Thinking workshop. Design Thinking is an innovation method for targeted solution finding, among other things, by structuring effective, interdisciplinary teamwork. The teams go through 6 phases within the framework of a challenge (thematic delimitation of a problem area), each accompanied by an experienced coach, to finally create an initial prototype (e.g., product, approach, concept) to solve the given challenge. In detail, during the first exercise, the participants perform interviews with different stakeholders. Thus, they get to know different perspectives and necessary information to uncover concrete underlying problems. 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. Beside the Design Thinking workshop, the Summer School will offer a platform to inform oneself about current 3Rs topics in various disciplines on an international level and their translational implementation, to learn about new methods and to network. Therefore, the program includes a presentation part with short talks by the participants and impulse lectures by relevant experts (e.g., Prof. Ellen Fritsche, Prof. Steven R. Talbot, or Prof. Doris Wilflingseder) from different fields and is completed by a variety of social networking events (e.g., beach volleyball, boat trip on the Spree), which offer valuable opportunities to get to know each other better and plan future collaborations in a relaxed atmosphere.

Here we will explain our concept, report our experience, and present the final prototypes generated during the Summer School in order to inspire other interested researchers or institutions to develop or provide comparable education and training opportunities. Our concept will guide scientists through a difficult and emotionally complex topic and aims at training scientists in teamwork, an open-mindset and creative confidence to facilitate a change within the scientific community including awareness for the 3Rs – towards *the next generation science with(out) mice*.

The ReThink3R 2.0 Summer School is funded by the Joachim Herz Foundation (funding scheme "Begegnungszonen") and Charité3R with additional sponsoring from the AniMatch UG (haftungsbeschränkt) and the Physicians Committee's Early-Career Researchers Advancing 21st Century Science (ERA21) program.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote required.

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3D lung-organoid platform for the identification of pharmaceutical compounds targeting senescence and inflammation

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Chronic obstructive pulmonary disease (COPD) and cystic fibrosis are hardly treatable or curable. Current treatment strategies are mostly symptomatic rather than causal or curative. Both diseases are characterized by chronic inflammation and degeneration of the airway epithelium resulting in loss of lung structure and function. Therefore, respiratory epithelial cells are an interesting target for therapeutic interventions. Here, we present our concept of a 3D lung-organoid-reporter platform for testing pharmaceutical compounds via High-Throughput-Screening (HTS) with regard to senescence, inflammation and toxicity. All measurements are performed in a 96-well format and easy to handle. SOPs with defined quality criteria allow testing under standardized conditions. We demonstrate that the pharmaceutical compounds quercetin and dasatinib counteract doxorubicin-induced senescence and modify inflammation. The platform will be useful for the thorough evaluation of numerous compounds (e.g., natural products) in pre-clinical studies and, most importantly, will reduce the need for animal testing.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote required.

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Integrated strategies for the design of core-shell structures as advanced barrier models

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Due to increasing pressure from the public and the limitations of current animal-based tests, the need for non-animal methods able to provide human-relevant results is emerging for drug testing and risk assessment of ingested and inhaled substances [1]. In this context, replicating the physiological 3D architecture and the dynamic mechanical stimuli of the intestinal and lung barriers is particularly relevant. Self-assembled spheroids and organoids are now emerging as powerful tools to replicate 3D structures. However, the spontaneous formation of an internal lumen is difficult to standardize and can be time-consuming [2,3]. As for the reproduction of the physiological mechanical stimulation, recent models based on deformable membranes are emerging. However, they utilize non-physiological stretching methods or do not respect the typical 3D physiological curvature [4,5]. Therefore, despite recent advances in *in-vitro* technologies, to date, a reproducible and controllable method for the fabrication of 3D dynamic biological barriers is missing.

Here, we present a new integrated *in-silico*/*in-vitro* strategy for fabricating 3D core-shell spheroids with predictable and controlled geometry, which can be actuated to mimic the physiological deformations of tissues as novel models of human barriers.

First, computational models were implemented in Comsol Multiphysics and Matlab to solve the equations of the drop formation and the hindered Ca^{2+} ion transport through porous media [6]. The models predicted the core-shell sphere geometry as a function of the extruded material, the extrusion parameters, and the crosslinking time to define the experimental working window. The best predicted results in terms of shell thickness were obtained with a core extrusion velocity of 10 µL/s, shell extrusion velocity 20 µL/s, and crosslinking time 15 min.

Starting from the working conditions predicted by the *in-silico* models, controlled formation of core-shell alginate drops was obtained using a COre-Shell MIcrobead Creator (COSMIC) equipped with a commercial coaxial needle (16-26 G). Preliminary tests were performed encapsulating Caco-2 (1.5 million/mL) cells in the shell, which was composed of alginate (20 mg/mL, A0682, Sigma Aldrich) and rat tail collagen (1 mg/mL, A10483-01, Gibco). The core was made of 10 mg/mL Pluronic-127. A 0.1 calcium chloride ($CaCl_2$) solution was placed under the needle to allow alginate crosslinking. Cell viability and permeability tests were performed using the Alamar Blue assay and quantifying the passage of FITC-dextran through the shell. The experimental results confirmed the shell thickness predicted by the *in-silico* model. Moreover, cell tests showed that the encapsulation procedure is cytocompatible. After 14 days of culture, dextran diffusion through the shell was reduced by 43% thanks to the presence of the cells: these results confirmed the barrier performance of the core-shell spheroids.

This *in-silico*/*in-vitro* approach represents a promising strategy for the fabrication of core-shell spheroids with defined geometry. In particular, the *in-silico* tools can be used to predict the effects of different core-shell material combinations, reducing experimental time and costs. Further investigations are currently ongoing to improve the model's physiological relevance, and future studies will include the core-shell spheroids embedding into physiological actuation systems.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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A new highly representative *in-vitro* model of human skin

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The human skin is characterized by a complex structure. Its main function is its protective role against exogenous substances, such as chemicals and immunological barrier.

Several *in-vitro* approaches are available to recreate a viable and functional model, in order to test the permeability of a drug or a cosmetic compound. Moreover, the inputs from the regulatory point of view are aligned o increase the use of alternative models, reducing the number of animal tests.

Therefore, it is possible to observe a wide variety of skin models aiming to recreate the complexity of the real tissue. Conventional mono-layered cell cultures are the basic and not so representative simulation step [1,2]. These models can be refined up to the more complex 3D co-cultures. In this context, the commercially available reconstructed tissues [3] are the closest "tissue" to the reality. These models are composed by human cells, cultured in a 3D structure which is topologically closed to the real skin. However, 3D micro-structures, such as sebaceous glands or hair bulbs, are not always reproduced. Moreover, they are developed and sold in transwell/inserts. In general, the focus is on the tissue topology and functionality, not on the possibility to recreate the suitable dynamic environment conditions to mimic the reality.

Is it possible to increase the predictivity of a skin model, contributing in the reduction of animal tests?

The use of human derived material, such as biopsy is for sure a useful step. This approach fills the lack of certain 3D micro-structures in some of the commercial tissue, in particular due to the presence of microstructures that in general don't characterize a reconstructed tissue.

However, the maintenance of a viable tissue, as well as its functionality are still a challenge.

In this context, the research group directed by Prof. Calderan (University of Verona, Italy) and IVTech srl [4], have collaborated to overcome this limitation [5]. Indeed, an advanced *in-vitro* model, involving the use of a human skin biopsy and a fluidic platform (i.e., LiveBox and LiveFlow produced by IVTech), is described in this work. In details, the skin biopsy obtained from patients is irrorated by a flow of medium, allowing for good results in maintenance of viability and functionality for a long-time experiment. The combination of a 3D biopsy and a dynamic environment has a key role in the increase the predictivity of an *in-vitro* model if compared with human reality. This is demonstrated, focusing the attention on 3 different cases of study: *in-vitro* administration of hyaluronic acid on a healthy tissue, and the characterization of 2 disease models, such as inflamed skin and skin affected by contact dermatitis. These results are the baseline to demonstrate the efficacy of the model, which can be used to test as a service the effects of drugs on a highly representative model of the human skin.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Bridging the gap between *in vitro* & *in vivo* testing: 3D cell culture taking into account the extracellular matrix

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The extracellular matrix (ECM) is present in all tissues and is a master regulator of cellular behaviour and phenotype: it influences the anchorage of the cells, acts as a migration barrier and track, signal reservoir, low-affinity co-receptor, signal presenter and is a source of biochemical forces. The ECM in each tissue/organ is characterized by a specific composition, and by biochemical and biophysical properties. Importantly, ECM features are modified in different types of disease, like cancers and fibrotic conditions [1]. Given the importance of the matricial microenvironment in maintaining tissue homeostasis and proper intercellular communications, we aimed at integrating the ECM parameter in *in vitro* models to better mimic the *in vivo* complexity.

For this purpose, we have developed 3D cellular models using BIOMIMESYS®, a patented *hydroscaffold*™ for 3D cell culture. This matrix exhibits unique dual properties, unifying hydrogel and solid scaffold features in a single matrix. This highly reproducible matrix is suitable for long-term 3D cell culture. BIOMIMESYS® is based on Hyaluronic Acid (HA), a major component of the ECM, biofunctionalized with other ECM components (collagens, adhesion proteins or peptides) depending on the organ/tissue of interest [2]. Moreover, the stiffness is also adjusted to fit with the healthy or pathological ECM to reproduce (elastic modulus from 0.1 to 16 kPa).

In this presentation, we will exemplify the importance of the matricial environment with the successful differentiation of human pluripotent stem cells (hiPSCs) into mature and functional 3D liver organoids within BIOMIMESYS®. This model provides a suitable model to study healthy liver functions but also metabolic diseases upon hepatocyte-like cell (HLC) differentiation [3]. Moreover, we will take the example of cancer tissues and how we can model the tumoral matrix with a representative range of stiffnesses (1, 8 and 16 kPa) *in vitro*. Finally, we will explain how BIOMIMESYS® can advantageously be used in various vessels: from multiwell plates (96-well format) for screening, to microfluidic chips for convenient, long-term dynamic *in vitro* modelling for both healthy liver and cancer settings.

By better mimicking the ECM microenvironment, the next generation of 3D cell culture models should help to model *in vivo* complexity with more relevance, and therefore to discover new effective therapies against diseases like cardiometabolic conditions and cancers. Our 3D *in vitro* models, by taking into account the matricial microenvironment, aim at bridging the gap between 2D *in vitro* models and *in vivo* situation.

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Submission declaration:
Conflicts of interest: All authors declare being employees of HCS Pharma.
Statement on ethics vote: There is a positive ethics vote.

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Cardioids unravel human heart development and defects

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The number one cause of fetal death are defects in heart development. Because the embryonic heart is inaccessible, impacts of drugs and environmental factors during pregnancy are unclear, and specialized functions of different heart compartments are not captured by *in vitro* models, determining underlying causes is difficult. Here, we established a human cardioid organoid platform that recapitulates the development of the major embryonic heart compartments, including right and left ventricles, atria, outflow tract, and atrioventricular canal. By leveraging both 2D and 3D differentiation, we generated homogeneous progenitor subsets with distinct first and second heart field identities. This advance enabled the successful generation of reproducible cardioids with compartment-specific *in vivo*-like gene expression profiles, morphologies, and functions. We used this platform to unravel the ontogeny of signal and contraction propagation between interacting heart chambers and to dissect how genetic and environmental factors cause region-specific defects in the developing human heart.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Antibiotics free nanoformulations for treatment of non-healing wounds

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Globally, over 1% of the population suffers from chronic non-healing wounds, negatively affecting patients' quality of life including low work productivity, immobility, amputation, lengthy hospitalisation, and death, costing several billions of dollars to manage or treat. Current wound care modalities rely on necrotic cells debridement, surgery, hyperbaric oxygen, antibiotics, nitric oxide, and wound dressing (such as hydrogels, alginates, and silver-impregnated dressing) [1]. Unfortunately, these options do suffer from various limitation due to lack of specificity and insufficient blood flow at wound beds causing delay in the wound contraction resulting in virulent infection and persistent inflammation [2]. Conventional antibiotics for treatment of infected wounds is not always effective due to growing antibiotic resistance.

Herein, we developed a nutrient based nanoformulations (NFs) of retinol, curcumin, PHMB when alone or in combination, with the former for tissue regeneration and later for antibacterial functionality. These NFs were characterised using dynamic light scattering to obtain the hydrodynamic particle (HDP) size and Raman spectrometer to obtain the vibrational bands of the NFs. *In vitro* skin penetration studies were conducted to assess the permeability profiles of the NFs. Synthetic membranes (Strat-M®; Merck, Germany) were used as a non-animal based skin model, mimicking the structural and chemical characteristics found in human skin. More so, the major components of the NFs were tested against *P. aeruginosa* and *S. aureus* for the evaluation of their antibacterial efficacy.

The HDP sizes of the NFs ranges from 9 nm to 14 nm with low polydispersity index of 0.08. Moreover, the NFs were stable over a predetermined time course. The retinol micelles were able to penetrate the membrane's surface, which is equivalent to the stratum corneum in human skin. In addition, the retinol is mainly retained and distributed in the upper layers of the membrane that is equivalent to the skin epidermis. The antibacterial efficacy of the major composition of the NFs against *P. aeruginosa* and *S. aureus* was demonstrated by the low minimum inhibition concentration (MIC) values obtained.

The preliminary data suggests that these NFs could be a potential formulation for treatment of non-healing wounds. However, further studies need to be conducted including comprehensive testing of the NFs' antibacterial efficacy, cellular viability, tissue regeneration and wound closure.

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Submission declaration:
Conflicts of interest: William Buchanan is the director of Phytoceutical Limited and he was involved in the conceptualisation of the work.
Statement on ethics vote: ethics approval is not applicable for this work

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Lush Prize 2012- 2022 and beyond: Supporting human-relevant, animal-free research

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This year, Lush Prize looks back over its first ten years of rewarding successful initiatives in science, training, public awareness and lobbying, as well as bursaries for young researchers across the world, all working to achieve the urgently needed paradigm shift towards modern, fit for purpose human-relevant science and away from animal use [1].

Looking ahead to November this year, winners of the 2022 Lush Prize will be awarded a further 290,000 EUR. This will take the total funding awarded since 2012 to almost 3 million EUR across the world. Winners continue to be awarded across five main categories for success; Science, Training, Public Awareness, Lobbying and Young Researchers. The first four categories are retrospective as they reward achievements over the last two years. The fifth category – Young Researcher Awards – are different as they provide individual bursaries to early career scientists for future animal-free research projects.

The Prize was set up to provide a new source of urgently needed funding and recognition for animal-free methods in safety testing and research. Despite positive steps in some areas, recognition and investment is still incredibly low worldwide. In the EU for example, funding for development of human relevant, animal-free methods remains consistently *well below 1%* of national government science budgets. Furthermore, the officially quoted figure of animals used in research each year across the EU is between 10 and 11 million, however taking into account all animals killed for their tissues or bred and not used before being killed, the more accurate total is over double this figure (23.5 million) [2].

Lush Prize continues to support the joint call for diversion of funds away from inadequate animal models to *better science* which combines the best methods in more quantitative and mechanistic approaches to answer research questions across both safety testing and basic research. Winning projects include development of human relevant *in vitro* and *in silico* methods including advances in Multi Organ Chip strategies, computational toxicology and Adverse Outcome Pathway (AOP) based approaches in toxicity testing. Training awards include successful projects to replace animal use in academia and "hands on" or virtual webinar training in New Approach Methodologies (NAMS) for the research community. In the Public Awareness and Lobbying categories, achievements awarded include reforming chemicals legislation and success in replacing animal tests with NAMs in specific areas.

The prize also continues to develop its "1R network" with the objective of connecting both former winners and new members to collaborate and share knowledge on the transition away from animals and towards NAMs.

All EUSAAT attendees and former prize winners are welcome to the Lush Prize booth to find out more about the Prize and the 1R network, or just to chat!

After this year's successful winners are announced in November, Lush Prize outreach and activities continue into 2023 and the next prize will be held in 2024. For more information, visit www.lushprize.org

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

165

3D-(bio-) printing for advanced *in vitro* systems to investigate nano-antibiotics against bacterial infections

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To study bacterial biofilm infections and novel ani-infectives, animals are still considered as a standard model in research. Animals are usually infected with bacteria loaded microbeads [1] via the intratracheal/intranasal route to investigate biofilm lung infections or intradermal to study skin and hair follicle infections. However, the clinical transitions of those experiments are questionable, because of interspecies differences (anatomy, genetics and immunology). Moreover, biofilm infections occur on top of epithelial mucosa, which is not simulated by biofilm growth in microbeads. Here we report two novel approaches as alternatives to animal testing via the support of 3D (bio-) printing.

Firstly, we established a method to bioprint bacterial biofilms for anti-infective testing and for the design of chronic infected *in vitro* models on human epithelial cells. Current biofilm *in vitro* models are limited with regard to biofilm reproducibility and design possibilities [2]. To overcome that, the objective was to pregrow biofilms in a hydrogel, which enables us to bioprint biofilms directly in any shape and dimension.

A self-established, characterized gelatin-alginate bioink was inoculated with E. coli MG1655. Biofilms were formed in a syringe for 3 days and were bioprinted afterwards in various shapes and dimensions. Imaging, antibiotic susceptibility assays and metabolic profile analysis confirmed biofilm properties when compared to bioprinted planktonic bacteria and native biofilm.

Secondly, we designed a novel 3D *in vitro* organ culture of human hair follicles. Conventional *in vitro* hair follicle models where single follicles are "free floating" in a medium [3] are not suitable for studying follicular penetration of nanoparticles. Thus, we provide a 3D model, with perpendicularly oriented hair follicles to be applied for penetration studies of nanocarriers [4], in particular for treating severe follicular diseases, such as *Acne Inversa* or *Folliculitis Decalvans*.

Human anagen hair follicles were isolated from skin biopsies originating from cosmetic surgery. Then, they were implanted perpendicularly into a 3D printed collagen matrix scaffold, emulating the dermis. Cultivability was assessed via hair growth in the model and compared to the conventional culturing method. Inserted hair follicles showed similar length increase on the respective days. To make a proof of concept, commercial nanoparticles were applied on the model to observe follicular transport. Preliminary experiments showed better follicular transport of 200 nm spheres to 500 nm within 4 h. To investigate treatment options the system can be drop-infected with *S. aureus*, with follicular colonization being confirmed by CLSM.

3D (Bio-) printing could facilitate future design of *in vitro* models to improve animal testing alternatives. Here, we showed a novel approach to bioprint bacterial biofilms to investigate anti-infectives against biofilm infections. On the long-term, we aim to bioprint these biofilms on human epithelial cells to mimic a chronic infection *in vitro*. Additionally, we showed a novel 3D infectable hair follicle model in a 3D printed scaffold system, which could serve as a potential platform to test topical transport systems against hair follicle disease related strains. A further analysis of the hair follicles' inflammation process after infection will be performed.

The authors would like to thank the SET foundation for project financing.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Increase the quality of animal research with CIRS-LAS – Research before the study, report during the study and discuss after the study

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The 3R principle constitutes the basis of animal studies. Therefore, there is a wide range of ideas and concepts to advance the principles of replacing, reducing and refining. The web-based database CIRS-LAS [1] addresses the areas of Reduction and Refinement by providing a platform to collect and analyze critical events that occur in the context of animal husbandry, animal breeding, and animal experimental research and to make them available to others working with laboratory animals. Use of the database has great benefits throughout the entire process of the study. Recorded incidents are researched or own incidents are added before the start, during the progress and after the completion of the experiments. This leads to a transparent publication of the data with the inclusion of a critical evaluation of the research.

How does it work? On www.CIRS-LAS.org, anyone can enter an incident report within a few minutes. For this purpose, keywords are requested to allow a later search in the database, as well as information on the animal species, the background of the experiment or general information, a short description of the incident and a classification in a subject area. If improvement measures have already been taken, these can also be indicated [2].

Currently, more than 250 registered users worldwide can search the database by topic, animal species or subject area in 50 reported cases. Searches in advance of a planned experiment can prevent the repetition of failed experiments and thus reduce the number of experimental animals. The quality of one's own work and published data increases because the findings from reported critical incidents can be taken into account.

Transparent publishing and an open-minded error culture with regard to critical events in animal research must be lived in every research facility and supported from the employee up to the management level. Both should be a part of daily work and a matter of course. The CIRS-LAS database and the optional anonymous case reporting are important tools to enable all persons working with laboratory animals to deal with errors in a transparent and open manner and thus contribute to improved animal welfare and a reduction in the number of laboratory animals.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote needed

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Turning AOPs into testable hypotheses with natural language processing and tissue-specific knowledge graphs

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The Adverse Outcome Pathway (AOP) framework is crucial to success of new approach methodologies for toxicity testing. To maximize the potential of AOPs by allowing scientists to easily turn them into testable hypotheses, we have developed a method to translate the English descriptions of each AOP component to unique ontology descriptors. This generates a fully semantic representation of an AOP, by easily mapping all its events to the most appropriate ontology concepts, also denoted by nodes in a massive knowledge graph (KG). Using a specialized artificial neural network model with pre-trained word embeddings and carefully curated pairs of similar/dissimilar biological event descriptions, we produce high-accuracy candidate ontology mappings for every molecular initiating event, key event and adverse outcome to a controlled vocabulary. For example, AOP events "protein alkylation" and "liver fibrosis", are mapped to Gene Ontology [1] concept GO:0008213 and Human Phenotype Ontology [2] concept HP:0001395, respectively.

Using *in vitro* transcriptomics data, and gene relations from a tissue-specific KG, we then calculate statistical significance of each AOP event and provide a transparent justification of which genes are involved in each event. After curating three OECD-endorsed AOPs quickly and painlessly using these mappings, we demonstrate a use case that takes advantage of our novel semantic AOP definition, showcasing how manifestation of an AOP can be queried using post-exposure transcriptomics data for known stressors. The experimental evidence becomes increasingly compelling when, if measured as a time series and the time points match the molecular dynamics of the toxicant, we can observe enrichment of AOP events shifting towards the adverse outcome over time. Furthermore, the KG used for AOP scoring is tissue-specific and expandable; and our AOP-mining approach is applicable to all types of 'omics (gene, protein, metabolite), *in-vitro* assays, and other platforms for NAMs.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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cellasys #8: A microphysiometric test to identify serum-free cell culture media

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Most Life Science labs culture cells using fetal bovine serum (FBS), which is collected from fetuses when pregnant cows are slaughtered [1]. It is estimated that the worldwide annual production of FBS is about 600,000 to 800,000 liters, collected from around 1 to 2 million fetuses. While this vast number of fetuses per year entails not only severe ethical issues, the usage of FBS is also associated with substantial quality and reproducibility concerns (e.g., unknown components that also vary from batch to batch) [2]. For these reasons, scientists are trying to transit from serum-based cell culture media (CCM) to chemically defined CCM without FBS. To do so, weaning experiments are usually carried out to evaluate new serum-free CCM formulations. Here, cells are gradually switched from a serum-based CCM to a serum-free alternative. However, such experiments take weeks to months [3], resulting in labor-intense and, hence, time-consuming tasks. To address this issue and foster the acceleration of serum-free CCM, the talk presents a methodology to identify serum-free CCMs as well as a case study with L929 cells.

Building upon our established microphysiometry technology [4], we implemented a standardized and automated testing schema, referred to as cellasys #8, designed to quickly identify the impact of serum-free CCMs on cellular metabolism and morphology. In detail, miniaturized pH, oxygen and impedance microsensors allow real-time measurements to identify changes in cell adherence and vitality. In combination with an automated and integrated medium change from serum-based to serum-free CCM, the test setup allows the evaluation of two crucial features: Firstly, the culture with a novel CCM is evaluated to identify its suitability and, secondly, the recovery phase is evaluated when cells are cultured again with serum-based CCM afterward. To demonstrate the applicability of the cellasys #8 test, we evaluated the suitability of three serum-free CCM formulations for the culture of L929 cells: (i) DMEM, (ii) DMEM/F12, and (iii) DME/F12 + ITS. DME/F12 + ITS was selected, as it is an established serum-free CCM for L929 cells. While the cellasys #8 test indicated changes in metabolism and morphology with DMEM and DME/F12, no changes were recorded during the treatment as well the recovery phase when cells were cultured with DME/F12 + ITS. This case study demonstrates that the cellasys #8 test is able to successfully identify serum-free CCM formulations at increased speed and reduced costs.

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Submission declaration:
Conflicts of interest: Dr Sebastian Eggert, Svitlana Stetsenko and Rainer Szalata are employees of cellasys GmbH. Dr Joachim Wiest is the CEO of cellasys GmbH.

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Immunocompetent 3D model of human airways as predictive tool for clinical outcomes

Martina Di Fede[1], Giulia Realini[1], Bruna Clemente[1], Serena Tondi[2], Marianna Taccone[1], Sonia Budroni[1], Martina Canè[3], Andrea Ariolli[4], Kevin Pete Buno[1], Francesca Schiavetti[1], Michela Brazzoli[1], Daniela Rinaudo[1], Isabel Delany[1], Raffaella Cecchi[1], Tommaso Pasquali[1], Shahjahan Shaid[5], Silvia Rossi Paccani[1] and Alfredo Pezzicoli[1]

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In vitro 3D models mimicking tissue microenvironment and complexity have attracted a great deal of attention. However, the successful implementation and acceptance of these models in pharmaceutical research requires a proper characterization and validation to demonstrate that they are fit for a particular purpose or context of use.

During the last year we have implemented a new 3D model of human airways, which includes innate immune cell components and a supportive fibroblast layer. With the ambition to induce a paradigm change in preclinical testing of novel candidates, we aimed to understand if this model could be used to physiologically reproduce human-pathogen interactions and predict clinical outcomes. For this purpose, we recently showed that specific proinflammatory cytokines associating with bacterial exacerbation in sputum samples of Chronic Obstructive Pulmonary Disease patients are secreted during non-typeable *Hemophilus Influenzae* or *Moraxella catarrhalis* infection of our immunocompetent models. We are currently completing this study by investigating if addition of vaccine-induced antibodies modulates inflammation phenotypes as it has been observed during clinical evaluation of the vaccine. All this work is pivotal for gaining confidence on the use of this model as possible predictive tool for clinical outcomes.

To further confirm the predictive value of the model, we are also investigating mechanisms of immunoglobulin passage across the respiratory models to understand if they can also mimic the physiological transepithelial transport on the respiratory mucosa. This aspect is of particular importance in the contest of development of Ig-based therapeutics and the use of these models could help to identify the best candidate able to reach the target site.

The application of complex *in vitro* models in current preclinical programs to assess efficacy of immune intervention in parallel to animal experiments may lead in the future to a partial replacement of animal models of infection, bringing new products to market in a cost-effective manner and operating in an ethical way.

Conflicts of interest: This work was sponsored by GlaxoSmithKline Biologicals SA which was involved in all stages of the study conduct and analysis. MDF, BC, MT, SB, KPB, GR, FS, MB, DR, ID, RC, TP, SS, SRP and AP are employees of GSK group companies. ST is a student at the University of Siena. MC is a student at the University of Naples – Federico II. AA is a student at the University of Tuscia. ST, MC and AA participate in a post graduate studentship program at GSK.

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Protein arginine methylation as a therapeutic target on patient derived autologous clear cell sarcoma model

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Clear Cell Sarcomas (CCS) occur extremely rarely and are regularly diagnosed in young adults. The patients receive a very poor prognosis due to diagnostic and treatment issues and early metastatic spreads. CCS are characterized by reciprocal translocation resulting in the expression of EWSR1-ATF1 fusion proteins. Besides this characteristic, CCS show no genomic peculiarities. Therefore, the search for new therapeutics in the epigenetic field is being attempted [1,2]. Protein arginine methylation (ArgMet) belongs to post-translational protein modifications and arises on many cellular proteins. Therefore, ArgMet plays a critical role in the epigenetic gene transcription regulation, signaling processes and RNA metabolism and subsequently controls proliferation, apoptosis, and survival. The enzymes that catalyze ArgMet are called protein arginine methyltransferases (PRMTs) [3,4]. PRMT overexpression has been implicated in various human cancers, and inhibition of PRMT activity can inhibit cancer cell proliferation and lead to cell death rendering PRMT inhibitors a possible effective therapy alternative to conservative treatments [5].

In the herein present study three different PRMT Inhibitors were tested on two recently established CCS cell lines originating from the same patient (derived from the primary tumor and a lung metastasis) showing elevated ArgMet levels compared to fibroblasts. Three-dimensional (3D) autologous co-cell culture technologies were established *in vitro*, to obtain the pathophysiological conditions of the tumor, to achieve a deeper understanding of CCS biology, and to gain fundamental insights into the response to PRMT inhibitors. After determining the half inhibitory concentration (IC_{50}) of each inhibitor, their effect was confirmed by ArgMet analyses using Nuclear magnetic resonance spectroscopy (NMR). In addition, apoptosis, cytotoxicity, and viability assays in two-dimensional (2D) and 3D cell culture have been performed.

The inhibitors used showed significant effects on used clear cell sarcoma cell lines in terms of viability and ArgMet. Interestingly, different effects were apparent in 2D compared to 3D and co-cultured cells, while no effect was detected in healthy cells. Our results indicate that PRMT inhibitors could be an appropriate novel treatment option for CCS patients with elevated ArgMet levels. The importance of patient-derived cell models in combination with advanced 3D cell culture experiments is obvious. The cell culture models used are an important step to reduce or replace animal models in the near future.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Evaluation of phototoxic potential and ability to form ROS of selected compounds by using EpiDerm™ phototoxicity test

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Evaluation of phototoxic potential of compounds and mixtures is a crucial step in the safety assessment of cosmetic, pesticide and pharmaceutical products absorbing UV and visible light. The validated and regulatory accepted *in vitro* assay, the 3T3 NRU PT (OECD TG 432), provides high level of sensitivity. However, it has been reported that this monolayer model also generates high rate of false positive results due to the lack of barrier properties naturally appearing in the human skin or other targeted complex tissues. *In vitro* reconstituted human skin models are increasingly being investigated for their usability in hazard identification and safety testing, because of their organotypic structure with a functional stratum corneum that allows for assessment of bioavailability of topically applied compounds and mixtures.

An *in vitro* phototoxicity test using the human reconstructed epidermis model EpiDerm™ (EpiDerm™ H3D-PT) has been developed and pre-validated almost 20 years ago and can be used either as standalone method for the phototoxicity testing of topically applied materials, or in combination with the 3T3 NRU PT, to minimize the potentially false positive results from this assay. In June 2021, OECD Test Guideline (TG) 498: *In vitro* Phototoxicity: Reconstructed Human Epidermis Phototoxicity test method was adopted. OECD TG 498 is a stand-alone method for evaluating the phototoxic potential of a test chemical after topical application in reconstructed human epidermis (RhE) in the presence and absence of simulated sunlight. EpiDerm™ H3D-PT is currently the only tissue model accepted under this test guideline.

The aforementioned method is based on evaluating the viability of exposed and irradiated tissues. However, another key determinant of photoreactive changes in cells is presence of reactive oxygen species (ROS). Therefore, we have selected a set of 13 compounds with known phototoxic potential and analyzed them using EpiDerm™ phototoxicity test. Moreover, we have studied the ability to form ROS using dichlorofluorescin diacetate assay (DCFH-DA) in combination with the UVA irradiation and the exposure to the test compound. Using this approach all 13 compounds were correctly identified as either having or not having phototoxic potential.

These preliminary results confirm that EpiDerm™ H3D-PT is a reliable test for the detection of phototoxicity and prediction of the phototoxic potential of selected substances and suggest that it can be also used to identify the formation of ROS after UVA irradiation. To confirm these results and to further examine potential of such combined method we will apply this approach to an expanded set of phototoxic and non-phototoxic compounds.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Identification and sub-categorization of ocular irritants using the EpiOcular tissue model – Prediction models for liquids and solids

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Determination of serious eye damage/eye irritation originally involved the use of laboratory animals (OECD TG 405). In 2015, a new test guideline (OECD TG 492) was accepted which enables the use of an *in vitro* procedure based on reconstructed human cornea-like epithelium (RhCE) to distinguish between chemicals (substances and mixtures) not requiring classification and those that must be labeled for eye irritation or serious eye damage. Chemicals identified as requiring classification for eye irritation/serious eye damage must be further tested to distinguish between eye irritants and those causing serious eye damage. There have been several projects focused on the development of tiered testing strategies for eye irritation assessment which takes in account all drivers of classification. The goal of these projects has been to develop a testing strategy to sub-categorize chemicals which: a) do not require labeling for serious eye damage or eye irritancy (No Category), b) can cause serious eye damage (Category 1 or Cat 1), and c) are eye irritants (Category 2 or Cat 2).

In the current project, a set of 13 chemicals (7 liquids and 6 solids) that are listed as proficiency chemicals in draft OECD TG 492B were tested using the RhCE model, EpiOcular. We used a testing strategy developed in CON4EI project and confirmed in ALT4EI project, which combines the most predictive time-points of EpiOcular time-to-toxicity neat and dilution protocols. Liquids and solids were test separately with different methodologies and prediction models. The set of chemicals consisted of 4 Cat 1 chemicals, 5 Cat 2 chemicals and 4 No Cat chemicals. Using the proposed testing strategy, we were able to correctly identify 100% of Cat 1 chemicals (4/4), 100% of Cat 2 chemicals (5/5) and 100% of No Cat chemicals (4/4).

The testing strategy proposed in CON4EI and verified in ALT4EI projects to achieve optimal prediction for all three categories – prediction models for liquids and solids seems to be a very promising tool in an integrated testing strategy (ITS) that can discriminate chemicals to No Cat, Cat 2 and Cat 1.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Development of physiologically relevant *in vitro* inhalation model to predict acute respiratory toxicity of mists and volatile liquids

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Acute respiratory toxicity (ART) testing is required to assess the health effects of inhaled substances. OECD accepted methods utilize GHS categorization that is based on animal death. There is no validated *in vitro* ART assay, even though animal tests have been discredited as predictors of human responses and on ethical grounds. The goals of this work were to develop physiologically relevant ART tests using the EpiAirway™ tissue model, demonstrate interlaboratory transferability, and correlate the results to an established categorization system relevant to human respiratory irritation.

Test articles (TA, n = 53) were applied to EpiAirway tissues (0.6 cm^2) at MatTek (USA) and IVLSL (Slovakia) with ART protocols developed for exposure to mists/sprays (Direct Application Protocol, DAP) and vapors/volatile liquids (Vapor Cap Protocol, VCP). In both protocols, tissues were exposed for 4 hours to 4 fixed doses of the TA (0.5, 2, 10, 20 mg/tissue, diluted in corn oil or water). In the DAP, TAs were applied to the apical tissue surface and in the VCP – to an absorbent material in a specially designed cap that forms a tight seal above the tissue allowing exposure to TA vapor. The effects on tissue viability (MTT assay) and barrier properties (Transepithelial Electrical Resistance, TEER) were determined. The effective doses which reduced tissue viability by 25% (ED-25) or by 75% (ED-75) were mathematically interpolated for the DAP and VCP methods, respectively. The ED-25 and ED-75 were correlated to the acute irritation Health Effects (HE) Codes (HE14/15/16) listed by OSHA, which are relevant to human exposure. Using the MTT assay, the DAP discriminated between HE14/15/16&NH with a Sensitivity/Specificity/Accuracy (S/S/A) of 77.6/87.6/82.6% (MatTek) and 75.5/86.1/80.8% (IVLSL); correlation to GHS Cat.1&2/3&4/5&NC gave results of 63.5/76.1/69.8% (MatTek) and 63.8/76.1/70.0% (IVLSL) S/S/A. The VCP discriminated between HE codes with S/S/A of 80.9/90.5/85.7% (MatTek) and 77.6/90.0/83.8% (IVLSL); correlation to GHS was 70.1/82.9/76.5 S/S/A (MatTek) and 71.1/82.2/76.7% (IVLSL).

Both protocols demonstrated high predictivity of human HE Codes, which are more relevant to human respiratory toxicity than the GHS categories. Good inter-laboratory reproducibility was observed for the VCP. The VCP and DAP provide physiologically relevant, organ-specific *in vitro* tests that can improve the predictivity of human responses and significantly reduce the number of animals being used.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Pluripotent stem cell assays: Modalities and applications for predictive embryotoxicity

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Developmental toxicology has long been considered an area of specific interest in the safety assessment of chemicals, pharmaceuticals, crop protection products and biocides, especially since the thalidomide episode in the early 1960s. This experience prompted the need for premarketing safety testing for embryotoxicity using rodent and nonrodent small mammals such as mice, rats and rabbits, to predict human safety. Worldwide regulatory animal study protocol guidelines were established by (OECD) since the early 1980s and have continuously been updated https://www.oecd.org/chemicalsafety/testing/oecd-guidelinestesting-chemicals-related-documents.htm).

We have reviewed the development of embryonic stem cell (ESC)-based models for alternative embryotoxicity assays. Against the wide spectrum of developmental toxicity, a huge diversity of alternative methods has been developed over the past half century. Among these the first one using pluripotent stem cells the mouse embryonic stem cell test (mEST) developed 30 years ago was the most promising one. It was based on the inhibition of cardiac muscle cell differentiation from mESC by test compounds as observed by scoring contracting cardiac foci under the light microscope. After successfully validation the mESC was standardized and published as Nature Protocol as NAM for predictive embryotoxicity in 2011 [1].

After this success, a variety of routes of embryonic differentiation and other readout systems have been assessed that broaden the applicability of EST to compound testing in PSC lines from diverse species including human hiPSC. Critical aspects including biological domain, readout endpoint, availability of standardized protocols, chemical domain, reproducibility and predictive power of each assay will be described, in order to review the applicability and limitations of the platform in general and progress moving forward to implementation. As collective experience increased with the use of PSC lines, derived either from mouse embryos (mESCs) or as established human cell lines, the strengths and limitations to predicting human safety have become apparent. Concomitantly, the reduction, refinement and replacement of experimental animals for toxicity testing (3Rs) is a major driver of the need for new approach methods (NAMs) to reliably identify developmental hazards and characterize their risk to healthy human pregnancy outcomes.

In order to add transparency and reproducibility to the review, systematic scoping review of the literature was conducted. Several formats and methodologies for literature review exist such as expert narrative review, systematic scoping review, systematic map, rapid systematic review, or full systematic review. Systematic reviews have been implemented in clinical research by Cochrane Collaboration over the last 40 years and brought comprehensiveness, objectivity, reproducibility and transparency to medicine and are the foundation of evidence-based medical practice [2]. On the basis of results from this broad scoping review of literature, the EST methodologies are discussed in the context of application for non-animal chemical and pharmaceutical safety assessment of prenatal developmental toxicity. This discussion includes relevance for human safety assessment, chemical applicability domains, and a path for international regulatory acceptance. Additionally, the list of included studies and chemicals that were tested in the assays may serve as a resource for OECD and other parties interested in the subject of developmental toxicity testing.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Evaluation of ocular side effects of systemically administered medications using the *in vitro* reconstructed human corneal epithelial tissue model

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Chronic use of systemic medications can cause light sensitivity, pain, corneal edema/inflammation, and/or cytotoxicity. Animal tests are often poor predictors of human responses. There is a worldwide need for physiologically relevant, human primary cell-based tissue models to address ocular safety for the evaluation of new drug formulations.

We have utilized an *in vitro* reconstructed EpiCorneal™ tissue model to analyze the effect of frequently used drugs with known adverse ocular side effects. EpiCorneal tissues are cultured using normal human corneal epithelial cells, express site-specific mucins and tight junctions, and attain morphology, barrier properties (Transepithelial electrical resistance or TEER > 1000 ± 200 Ω*cm^2), and gene expression comparable to the *in vivo* human cornea. Tissue performance, evaluated by TEER and tissue viability (MTT assay), were comparable after 24 h and 96 h under simulated shipping conditions.

The effects of Chlorpromazine hydrochloride (CPZ), a common psychotropic agent; Hydroxychloroquine sulfate (HCQ), an anti-inflammatory/anti-malaria drug; Alfuzosin hydrochloride (ALF), antihypertensive drug; and Fosamax (Alendronate Sodium, FOS), a common anti-osteoporosis agent, were investigated. Endpoints included MTT, TEER, histology, and LDH and cytokine release. Tissues were incubated in the medium containing physiologically relevant concentrations of the drugs for 24 h and 48 h. For CPZ-treated tissues, the lowest dose to cause a significant decline in barrier function (67.4%) was 12.5 µM at 24 h; 25 µM decreased tissue viability (60.5%) at 48 h. For HCQ-treated tissues, a decline in TEER (67.4%) was detected for 18.52 µg/mL at 24 h, and in viability (53.6%) for 55.56 µg/mL at 48 h. For ALF-treated tissues, major declines in TEER and viability were observed at 500 µg/mL after 24 h, while an increase in IL-8 release – at 0.005 µg/mL at 48 h. For FOS-treated tissues, a significant TEER decrease (57.8%) was detected at 0.1 µg/mL and in tissue viability (85.7%) at 10 µg/mL at 48 h. Treatment-specific changes in tissue morphology and dose response of LDH were also observed.

EpiCorneal tissue model is valuable for evaluating formulations with negligible irritation potential. It is suitable for rapid drug screening, will model systemic and topical drug exposure, improve the predictivity of human responses, be more cost effective and reproducible than animal methods. It will facilitate drug discovery worldwide by allowing screening and optimization of pharmaceuticals prior to clinical studies.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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High throughput intestinal tissues on newly fabricated 96-well culture plates

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High throughput 96-well tissue culture plates that allows culture of three-dimensional physiological tissue systems of small intestinal (SMI) organotypic tissue on a newly fabricated membrane support was developed. The plates were used to reconstruct human primary intestinal tissues and cultures were followed for over 3 months by monitoring tissue histology and barrier integrity as measured by transepithelial electrical resistance (TEER). Histological specimens were collected on weekly basis. To monitor well-to-well tissue reproducibility, TEER measurement was performed every week on all 96-well tissues. Histological and immunological stains showed that tissues fixed at all time points (> 3 months) were structurally similar to the standard EpiIntestinal tissue structure and morphology. The 3D tissues are well polarized and stratified with villi like structural formation. Immunohistochemical staining also shows epithelial marker (CK19), tight junction formation (ZO-1), and brush border marker villin similar to the standard tissue model. The barrier integrity measurement (TEER) demonstrated high well-to-well reproducibility (weekly average %CV < 20%) for up to 90 days of the culture period. The weekly measurement of TEER for all the wells/tissues were within a physiological range of 160-300 ohms*cm^2. This value was within our QC criteria for a viable EpiIntestinal tissue model. Availability of intestinal organotypic tissue that can be cultured for extended period of time (3 months) can be used for chronic exposure experiments. This result extends our previously published tissue utility time point from 42 days to 90 days. In conclusion, the newly fabricated plates support reconstruction of EpiIntestinal tissues for extended time period that can be used for hazard identification of chemicals and nanoparticles in a high throughput format and to study drug safety and efficacy following chronic exposures or multiple applications of compounds.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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An epidermal model containing melanocytes for skin pigmentation and lightening studies

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Considerable interest exists in evaluating raw materials and/or skin care formulations which cause lightening of the skin. These products are utilized to modulate one's natural skin color or to combat skin pigmentation disorders such as melasma, dark spots, solar lentigo, and other hyperpigmentation lesions. To aid in the development and testing of such products, we have developed a skin whitening protocol using the epidermal skin model, MelanoDerm™, to evaluate both raw materials and skin lightening formulations. MelanoDerm is a highly differentiated, three-dimensional tissue culture model of human epidermis that contains normal human melanocytes (NHM) and keratinocytes (NHK). Epidermal tissues have been produced containing NHM of varying skin phototypes which follow the pigmentation level of the donor tissue, i.e., black > Asian > Caucasian. For lightening studies, tissues were treated topically three times a week over a two- to three-week period to mimic consumer application. Several over-the-counter skin lightening products were evaluated in cultures containing NHM from black and Asian donors. Over the treatment period, negative control cultures became increasingly pigmented with retention of normal epithelial morphology. In contrast, tissues treated topically with cosmetic skin lightening agents containing tyrosinase inhibitors such as kojic acid and magnesium ascorbyl phosphate remained lighter than the control cultures. The skin lightening effect on treated tissues was quantitatively evaluated for melanin content using a Solvable melanin assay and for skin brightness (L* value) using a hand-held spectrometer. Treated tissues showed significant changes in overall melanin content and brightness compared to control tissues. These results suggest that this model can provide valuable *in vitro* data for screening raw materials prior to the commencement of costly clinical trials and that it will be useful to study melanogenesis, skin lightening, and other pigmentation phenomena of the skin.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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OrbiTox – Interactive visualization of multi-domain experimental and predicted data for translational discovery

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Large amounts of costly experimental data being generated across life, pharmaceutical, and chemical sciences are housed in databases such as DSSTox, PubChem, ChEMBL, DrugBank, TOXNET, HSDB, BARD, YummyData, UniProt, NCBI, KEGG, Reactome, GO, etc. In the absence of collective visualization and analyses of multi-domain data, it is arduous for an investigator to unearth new knowledge embedded in connections across these disparate data sets. Our interactive tool, OrbiTox, addresses this unmet need by displaying in a single, 3D, immersive environment not only large amounts of data from chemistry, gene targets, biological pathways, and experimental species but also connectivity across them.

Currently, OrbiTox contains curated data from 900,000 substances, 22,000 annotated human targets, 1,500 biological pathways, and over 100 test organisms. Experimental toxicity data are stored as 400,000 connections that represent human and rodent carcinogenicity, bacterial mutagenicity, acute toxicity, and various bioactivities.

In addition, OrbiTox houses a panel of QSAR models for over 40 Tox21 assays and bacterial mutagenicity models, with and without metabolic activation, in five OECD-recommended *Salmonella* and *E. coli* strain groups.

All models are based on our recently published *Saagar* descriptors [1] to substantiate each *in silico* prediction with chemistry-backed reasoning. In a similarity-search test involving ~145,000 compounds across 78 screens, *Saagar* proved to be more efficient in active enrichment than commonly used fingerprints like ECFP4, PubChem, MACCS, and ToxPrint.

The connectivity among data in OrbiTox allows querying and filtering of data from any domain. In one test study, we were able to query the fructose metabolism pathway, identify member genes of that pathway and identify inhibitors of a member gene AKR1B1 with their IC_{50} values – all in one view. Similarly, for any given chemical query, OrbiTox can fetch data-rich, chemistry-aware similar chemicals for read-across applications using *Saagar* substructures.

A unique OrbiTox functionality affords to map paths between inter- and intra-domain objects via connections through experimental data or predicted relationships to help hypothesize functional or mechanistic relationship between connected objects (chemicals, genes, or proteins).

The technology underlying OrbiTox allows real-time interaction with the data and is readily scalable to millions of data points allowing addition of proprietary data and predictive models.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Short- and long-term patient-derived sarcoma models – To study the variety of soft-tissue tumors

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Sarcomas only represent 1% of all adult malignancies and encompass over 50 different subtypes based on their molecular and pathological features. They originate from a diversity of mesenchymal tissue lineages including muscle, cartilage, bone or fibrous and can therefore occur in almost all organs. Most common histological subtypes include liposarcoma, chondrosarcoma, osteosarcoma and Ewing sarcoma, with the latter two being primarily pediatric tumors. Up to date, efficient therapy options have been limited due to only a small proportion of these types having specific diagnostic markers such as genes mutations or chromosomal translocations, with even fewer possessing identified molecular therapeutic targets [1,2]. A main obstacle for drug development and subsequent preclinical trials portrays the lack of suitable sarcoma *in vitro* models due to the rare incidence of each sarcoma subtype [3].

In general, continuous tumor cell lines are an important asset in cancer research as they are easy to handle, cost effective and give consistent data. Especially for pre-clinical studies, continuous cell lines are necessary for understanding the underlying molecular mechanisms of a given tumor cell and investigating the tumor suppressive effects of potential drugs. However, they do not necessarily resemble the *in vivo* situation and often require animal experiments in order to verify obtained data. Using primary cells, directly isolated from tissue, can mimic the tumor microenvironment more precisely by ensuring genotypic and phenotypic stability and effectively capture the complexity of sarcoma cells.

In this study post-surgical tumor tissue from a multitude of sarcoma entities including myxofibro-, chondro- and liposarcoma, was enzymatically and mechanically dissociated and the isolated primary cells further cultivated in appropriate media. In addition to the tumor tissue, surrounding tumor tissue and/or healthy skin tissue was collected of the same patients for isolation of cancer associated fibroblasts (CAF) and normal human dermal fibroblasts (NHDF), respectively. The obtained patient-derived primary cells underwent extensive quality control including mycoplasma analysis and short tandem repeat (STR) profiling for cell authentication. Morphology of the obtained patient-derived primary cells was observed microscopically and the cells stained for, if available, typical sarcoma markers. Using flow cytometry cell cycle analysis of the sarcoma cells and staining of cell surface markers were performed for further cell characterization. Cells were periodically frozen away and most promising primary cells were further passaged with the aim of establishing novel sarcoma cell lines.

The collection of primary cells derived from cancerous and healthy tissues of the same patient and the corresponding cell lines ultimately yields a master cell bank and the possibility to establish autologous sarcoma models, which imitates the *in vivo* situation reliably with the potential of eliminating the need for animal experiments altogether.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Cultivation of human skin biopsies for preclinical testing

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The current commonly used skin models to replace animal testing are build up models using isolated skin cell types (mainly keratinocytes and fibroblasts) as components resulting in skin equivalents with different levels of complexity. These skin equivalent models have gained wide acceptance to be suitable for acquiring knowledge about skin reactions upon exposure to different agents/conditions. Nevertheless, these models can never fully mimic normal *in vivo* skin, with its diversity of cell types (including resident immune cells) and nature of constitution. Consequently, the ideal model for making predictions would be adequate cultured *ex vivo* skin itself, to enhance the translatability of research.

In our lab we aim to offer cultivated *ex vivo* skin for research purposes. Skin biopsy punches from healthy foreskin operations, mostly from circumcisions with ethical approvals 28-476ex15/16 and 26-219ex13/14, are cultivated in transwell inserts. To preserve the skins natural environment, these punches are kept outside the incubator in CO_2-independent media. Cultivation of air-exposed epidermis and media supplementation from the dermal side through the insert's membrane allows applications not only from the topical epidermal side, as already shown by other groups [1], but also from the dermal internal side through addition of substances in the culture medium. For defined application of testing substances onto the epidermal side of the skin (e.g., creams, chemicals) 3D printed teflon rings restrict the area exposed to the surface.

Besides the viability of the skin organ culture, the integrity of the skin structure is likewise important during the whole time of the experiment to avoid artificial results. H&E staining provides a quick check to verify viability and skin integrity criteria [2,3]. Skin integrity is impaired visually by spongiosis (cleft formation within the epidermis) and acantholysis (loss of keratinocytes connection resulting in a draft between skin layers).

The *ex vivo* skin model can be used to assess new therapeutic approaches for the treatment of various skin diseases. Complex settings such as the interaction of a probiotic gel containing a population of *Lactobacilli* counteracting skin inflammation during neurodermatitis caused by bacterial colonization by *Staphylococcus aureus*, can be assessed in preclinical trials. Evaluation of the effect on *ex vivo* skin consists, among other things, in the analysis of specific cytokine release into the surrounding media, which corresponds to the predictions of the skin equivalence models [4].

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Updates of the EU3Rnet

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EU3Rnet is an open network for European 3Rs centers and platforms. The 3Rs centers and platforms are very important points of contact and play an immense role in their respective countries as "on the ground" facilitators of *Directive 2010/63/EU*. They are also invaluable for the widespread dissemination of information and for promoting implementation of the 3Rs in general [1]. In this contribution, the development of the EU3Rnet is presented and the history of the European 3Rs centers is recapitulated. A further focus is set on the special features, but also the commonalities of the 3Rs centers and platforms, especially with regard to organization, structure and topics. The first EU3Rnet consensus statement and the publication "The rise of Three Rs centres and platforms in Europe" are presented as previous highlights [1,2]. Recently, the COST Action IMPROVE with the topic "3Rs concepts to improve the quality biomedical science" was approved, which will strongly support the networking in the 3Rs area. This activity will be presented in detail with its possibilities, plans and instruments. The working groups will focus on Quality and Translatability of Science, Implementation, Dissemination and Education, whereby ethics will be an integral part in the work of the single working groups. Cooperation and active involvement in the COST Action will be invited within the framework of this open bottom-up network approach.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is no ethics vote applicable.

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Human platelet lysate as a replacement for fetal bovine serum in biological barrier *in vitro* models

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Introduction: Xenofree human cell culture is advantageous in view of scientific (batch variations, side effects caused by animal products) and ethical concerns, addressed by 3Rs (replacement, refinement, reduction of animal experimentation) societies, animal welfare organizations, and politics (Directive 2010/63/EU). Aside animal experimentation *per se*, the replacement of animal products in *in vitro* experiments – particularly fetal bovine serum (FBS) – is an important goal. One alternative to FBS in cell culture is human platelet lysate (hPL), a by-product of transfusion medicine.

Aims: In this study we evaluated the use of hPL in context of a human blood-brain barrier (BBB) and an oral mucosa epithelium model, based on cell lines hCMEC/D3 and TR146 respectively. hPL was compared to FBS regarding support of hCMEC/D3 and TR146 proliferation (short- and long-term cultivation) and barrier properties in a transwell model setup. In addition, a murine brain endothelial cell line, cerebEND, was included to test the suitability of hPL in non-human cell culture.

Methods: Proliferation of cells cultivated in media containing FBS or hPL (O-in-AB) were compared in growth curves and cell cycle analyses (flow cytometry). Measured barrier properties of cells grown on transwell inserts included trans-endothelial electrical resistance (TEER) and carboxyfluorescein permeability. ABCB1 activity was determined by Calcein AM uptake upon inhibition of the transporter with Verapamil. Gene expression profiling was performed by Fluidigm high-throughput qPCR. Expression and localization of marker proteins were shown by immunofluorescence microscopy.

Results: Cultivation of hCMEC/D3 and TR146 over 10 consecutive passages in hPL showed that proliferation is well supported by hPL, but at lower level as compared to FBS, which is also reflected in cell cycle distribution. The BBB and oral mucosa models revealed that both, cells grown in hPL and FBS, reach similar TEER values. The permeability coefficient for carboxyfluorescein was in both cases ca. 3 µm/min. Interestingly, the uptake of Calcein AM upon Verapamil treatment was higher in cells grown in hPL than in FBS, suggesting enhanced ABCB1 activity. Gene expression profiles were comparable, as well as expression and localization of marker proteins. Cultivation of murine cerebEND cells was not sustained by hPL media, after few passages morphological changes and cell death were observed.

Discussion: Our study shows that hPL is an adequate alternative to replace FBS in hCMEC/D3 and TR146 cell culture, but not in murine cerebEND cells. Most of the evaluated BBB and oral mucosa parameters are not changed in cells cultivated with hPL, in case of ABCB1 the transporter activity in the BBB model might be even enhanced by hPL.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Risk assessment of medical devices for the oral cavity using a human oral mucosa epithelium model

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Medical devices (MDs) have invaluable roles in today's healthcare. MDs used in oral cavity can be defined as a broad range of products, from creams and ointments for treating sores or aphthae, to dental adhesives and materials used in dental prosthetic devices. In the oral cavity, these MDs get into direct contact with oral mucosa and must be assessed for safety. Bio-compatibility assessment is usually still conducted in animals, however alternative *in vitro* methods are now available due to development of cell and tissue engineering, for example *in vitro* reconstructed epidermis test for intra-cutaneous testing (ISO 10993-23). These tests are typically based on testing toxicity of highly diluted extracts from MDs, where potential irritants are highly diluted and there is a need to fine-tune *in vitro* tests to achieve higher sensitivity and specificity for a certain type of MDs tested.

We tested different MDs used in the oral cavity on an oral mucosa epithelium barrier model based on buccal carcinoma cell line TR146. Cells were seeded on transwell inserts and cultivated under airlift conditions as recently optimized and published by Lin et al. [1]. The MDs selected to test are commonly used in oral cavity: cream for mouth ulcers, adhesive creams, tablets for treating gingivitis and different materials such as metals with different surface coating that are used in dental implants. Materials were weighted following the ISO guidelines (ISO 10993-12) and extracted for 24 h (creams and ointments 0.5 g/15 mL serum-containing basal growth medium, metal dental materials 0.2 g/mL serum-containing basal growth medium, boiled and autoclaved silon was weighed and filled with serum-containing basal growth medium to 0.1 g/mL), tablets were dissolved and diluted into used concentrations. After four weeks of cultivation and prior to adding the extracts, medium was added also on the apical side and after 30-minute calibration, the transepithelial electrical resistance (TER) was measured to assess barrier tightness. The apical medium (saliva compartment) was then exchanged with prepared extracts from MDs and incubated overnight. After 20 h incubation TER measurement was repeated, followed by a permeability assay with fluorescein (F) and lysis of the cells for further molecular analysis by high-throughput qPCR on the Fluidigm Biomark platform [2-4]. Permeability coefficients (PC) were calculated as previously published [1].

Results revealed detrimental effects on barrier tightness by the cream for mouth ulcers, whereas adhesive creams and dental materials made from titanium with and without different surface treatments did not have such significant effects. Moreover, extracts from tablets for the treatment of gingivitis seemed even to improve barrier tightness relative to controls. The TR146 oral mucosa barrier model was shown to be an appropriate model for testing the MD extracts, since it is responsive to the treatment with MD extracts, but was still stable and not overresponsive, thereby not leading to complete functional barrier loss. During the studies it became apparent that for adequate comparisons the controls and the treated cell layers have to possess similar barrier properties at the beginning of the experiment. In addition to the functional endpoints TER and F permeability, the TR146 model provided also the possibility to analyze the response to dental MDs at the mRNA level by high-throughput qPCR measuring up to 96 biological barrier specific target molecules.

Supported by the APVV grant DS-FR-19-0048 (Slovak Republic), MŠMT grant 8X20026 (Czech Republic) and OEAD grant MULT 06/2020 (Austria) in the course of the TraiNSafe-MD project coordinated by Helena Kandarova.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is no ethics vote applicable.

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Interaction and transport of small extracellular vesicles across human biological barrier *in vitro* models

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Introduction: Biological barriers (BBs) are selective physical, chemical and biological barriers between organs and outer compartments. BBs are crucial in maintaining homeostasis via selective transport of substances and cell-cell communication signals such as small extracellular vesicles (sEVs) [1]. sEVs are released by all cells, are found in different body fluids such as saliva (used as biomarkers) and have been reported to carry active molecules that can elicit responses in recipient cells [2]. The blood-brain barrier (BBB) is a very selective barrier that protects the brain from environmental toxins and pathogens and has been extensively studied in the drug delivery field [1]. The blood-saliva barrier (BSB) has been studied and modelled in the recent years to better understand the salivary biomarkers and mechanisms behind their crossing from blood to saliva [3]. Its sealing component are epithelial cell layers of oral mucosa and salivary glands [3], while BBB consists of brain capillary endothelial cells (BCECs) [1].

Aims: Small EVs can cross different BBs, even the blood-brain barrier (BBB), but the exact mechanisms are not elucidated yet. Therefore, we aim to study the interaction and transport of sEVs across the BBB and BSB

Methods: To model the BBB, immortalized cell line hCMEC/D3 and human induced pluripotent stem cell-derived brain capillary endothelial-like cells (hiPSC-BCECs) were applied, whereas for the BSB, cells of clone HTB41 B2 was used. BB models were established on 24-well transwell inserts (ThinCerts, Greiner). Initially, small EVs were isolated with combination of ultrafiltration and size exclusion chromatography from conditioned serum-free medium of donor cells; characterized with nanoparticle tracking analysis (NTA) for particle count and size distribution and with microBCA followed by Western Blotting for typical sEV enriched proteins (CD81, Alix, CD9) and a negative sEV marker (GM130). Furthermore, sEVs were fluorescently labelled (directly with transfection or indirectly with membrane permeable dyes, e.g., CellTracker Orange (CTO)) to enable later detection with fluorescence NTA and microscopy. To study the uptake and transport of sEVs, they were applied to the compartment corresponding to apical or basolateral side of the transwell model for 24 h-48 h. Transepithelial/transendothelial electrical resistance (TEER) of the cell monolayer was measured before and after applying sEVs.

Results: Control set-ups revealed a general, serum-dependent, but not EV specific reduction in TEER after 24 h and 48 h sEV incubation using hCMEC/D3 cells and HTB41 B2 cells. 1321N1 astrocytoma EVs added to BBB cells resulted in a decreased TEER. Permeation of sEVs across tight barriers was restricted: less than 10% of fluorescently labelled sEVs could cross the blanks, and even less went through the BB cells. The sEV uptake was evaluated with fluorescence microscopy that revealed membrane surface binding or internalization of CTO labelled sEVs in hCMEC/D3 cells using fluorescent markers such as EV-(e.g., CD81) endocytic pathway-related (e.g., Rab11) antibodies. Besides, flow cytometric analyses also confirmed the interaction between CTO labelled sEVs and BB models by shifting the peak of the fluorescence curve. An important part of our experiments were specific controls that allow us to follow good cell culture practice such as blank control studies of EV transport across inserts and microscopy of BB cells to confirm appropriate expression and organization of essential BB junctional proteins.

Discussion: Current *in vitro* models are limited to mimic the complexity of biological barriers; however, they can be sufficient substitutes for initial assessment of communication and transport between BBs and substance of interest. With particular care regarding reference points (EV specific controls for harvesting, isolation and labelling), we were able to reliably determine the uptake of EVs to specific BB cells. Our specific focus was laid on reproducibility of the data and validation of these models since this is essential for *in vivo* translation.

This work was founded by the European Union's Horizon 2020 research and innovation programme (Marie Skłodowska-Curie project No 860303). We further gratefully acknowledge the financial support provided by the Austrian Science Fund FWF (project P 34137-B)

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is no ethics vote applicable.

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Effects of nanoplastics on cells of the neurovascular unit

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The exposure of humans to nano-and microplastic particles (NMPs) is an issue recognized as a potential health hazard by scientists, authorities, politics, non-governmental organizations and the general public. The concentration of NMPs in the environment is increasing concomitantly with global plastic production and the usage of plastic materials [1]. In contrast to the very distinct media presence of NMPs, the actual state of knowledge about the effects of NMPs on human health is very low. There are several difficulties that need to be overcome in order to make a meaningful risk assessment of the effects of NMPs on human health. So far, a comprehensive risk assessment of NMPs is hampered by limited availability of appropriate reference materials, analytical obstacles and a lack of definitions and standardized study designs [1]. In addition, the interactions of NMPs with cells depend on the type of NMP, size, shape, surface structure, charge, polarity, etc. Moreover, the role of NMPs as a carrier material for toxins or microbiota needs to be investigated very fundamentally, and the effects of possible leaching of supplemented additives of NMPs have to be clarified and their relevance assessed. Recently, Leslie *et al.* have shown that plastic particles were found in the human bloodstream with a concentration of about 1.6 µg/mL [2]. The identified particles exhibited sizes below to 0.8 µm and counted mostly to less hydrophobic plastics such as PET ([poly(ethylene terephthalate)] or PS (polystyrene), but also some PP (polypropylene) or PE (polyethylene) was found. In relation to this finding, we decided to investigate effects of nanoplastics on the blood-brain barrier and surrounding cells of the neurovascular unit. Alterations of the blood-brain barrier are involved in a myriad of diseases such as Alzheimer's disease, multiple sclerosis, Parkinson's disease, epilepsy, etc., some of which are even causative.

Therefore, the aim of the study was to investigate the effects of different nanoplastic types on human brain endothelial cells, astrocytes and pericytes. PET, PP and PS were obtained from different providers and were produced by different methods. PET nanoparticles were produced by laser ablation [3], whereas PP nanoparticles were obtained by an own developed milling process. The PS nanoparticles (pristine, carboxylated, amino-modified surface) were commercially purchased from Polysciences Ltd. All particles were characterized for their average diameter, concentration and zeta-potential using nanoparticle tracking analyzer Zeta-View Quatt (Particle Metrix). Nanoplastics were in a range between 100-200 nm diameter and were applied on cells in different concentrations. After 48 hours of incubation cell viability (XTT-based EZ4U or MTT) was measured. In addition, brain endothelial cells were cultivated on transwell inserts. After nanoplastic treatment changes in transendothelial electrical resistance (TEER) were recorded, permeability of paracellular marker FITC-dextran 4000 was determined and cells were lysed for subsequent molecular analysis by high-throughput qPCR applying the Fluidigm Biomark platform [4,5]. Results showed that only concentrations significantly higher than the found ones in blood led to reduced cell viability (e.g., 30 µg/mL PET, 300 µg/mL PS) or decreased barrier integrity. Notably, high concentrations of PS led to turbid solutions which required specific adaptations and additional control approaches for the methodological read-outs. In summary, our data do not confirm that the tested nanoplastics at the concentrations found in blood exert harmful effects on the tested cells after acute treatment for 48 hours. Future experiments over much longer periods in fluidic models should provide information on possible effects of chronic exposure.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is no ethics vote applicable.

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Leaky gut model for the investigation of productive delivery of JAK-1 siRNA

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Antisense oligonucleotides profit from their high selectivity, however, are usually species-specific. Therefore, animal experiments often require the design, synthesis, and evaluation of a suitable extra molecule with a comparable function. Different organ physiology and disease manifestations and variations in immune response complicate the use of mouse models and compromise their predictivity for human therapy. Selection of ASO candidates and nanocarriers for productive delivery (= cellular uptake leading to the target pharmacological effect), can be performed using human cells. The multiple barriers to colonic delivery, however, are thus challenging that cell culture productive uptake mostly cannot be reproduced *in vivo*. Improvement of productive delivery needs to identify shortcomings like lacking penetration of mucus, instability in colonic fluid, inability to access immune cells, or insufficient endosomal escape. The *in vivo* experiment is suboptimal to spot the limiting parameters due to the needed high analytical effort. Simple *in vitro* assays may investigate each parameter isolated, however, the effectiveness of the overall approach is not easy to predict from a series of such experiments. In consequence, ASO delivery-technology research would profit from complex cell models, which may combine some most substantial barriers to allow the evaluation of productive delivery into human cells.

We developed a model of the inflamed intestinal mucosa containing epithelial (Caco-2), macrophages or macrophage-like cells (differentiated THP-1), and dendritic or dendritic-like (MUTZ-3) cells [1,2]. The presence of the innate immune cells increases the reaction to an inflammatory stimulus by cytokine secretion and a decrease in transepithelial electrical resistance compared to a Caco-2 monoculture. Such inflamed conditions, however, are not allowing nanocarrier access to the subepithelial immune cells. Seeing monocytic cells with nanocarrier uptake in the *in vivo* inflammation model, we adapted the coculture model to a condition of controlled leakiness by using it at a slightly sub-confluent epithelium. Model characterization included the viability assessment of the three cell types and cytokine secretion. The model was used to investigate lipid nanocarrier-mediated productive delivery of Jak1-siRNA *vs* transfection reagent Lipofectamine. Nanocarrier and siRNA localization was investigated using confocal laser scanning microscopy. The JAK-1 activity was measured as a downstream signal by STAT-1 phosphorylation by an antibody-based flow cytometry assay, showing the lipid nanocarriers as the more efficient delivery system [3].

Utilizing human cells, we aim to avoid species-related issues for translation. Progress in such multicellular, complex cell models representing the microenvironment of pathophysiology in acute or chronic inflammation may allow studying the disease and test actives and medicines for treatment.

This research was funded by the EU Horizon 2020 research and innovation programme under grant Agreement N° 720905 entitled NEW DEAL.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: not applicable.

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Modelling biological barriers for improving the delivery of novel anti-infectives

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Not ignoring the current pandemic, the threat of antimicrobial resistance might be even worse, and the problem is still increasing. Ironically enough, the number of new antibiotics and even the number of companies engaging in those is decreasing. Besides the need for new targets and actives there is also a need to deliver those across biological barriers. Besides the body's outer epithelia, like e.g., gut, skin and lung, there are also the bacterial cell envelope itself, as well as non-cellular barriers, such as mucus and bacterial biofilms.

Because of their complex and asymmetric structure, the Gram-negative bacterial envelope represents a very tough barrier for novel anti-infectives with intra-bacterial targets. Rebuilding the structure of this barrier on a Transwell® setup, we found some encouraging predictivity for actual *in bacterio* uptake and bioavailability [1,2], which we are currently further improving by using 3D-printed polymeric hydrogels [3], and by coating membranes using bacterial outer membrane vesicles (OMV's) [4].

When applied to the skin, nanoparticles do not cross the stratum corneum, but may penetrate into hair follicles to deliver antigens noninvasively across the intact skin barrier. In mice, we could demonstrate a significant cellular and humoral immune response to the model antigen ovalbumin [5,6]. But as the capacity of this pathway is limited, complementary technologies, like e.g., mRNA vaccines, are still needed [7,8]. Nevertheless, this pathway holds interesting perspectives for the targeted delivery of nanocarriers for the treatment of hair follicle diseases, such as alopecia areata or allergic dermatitis [9,10]. Ongoing activities include the development of a human hair follicle models based on 3D bioprinting.

To study the cellular interactions of drugs and nanoparticles after deposition in the deep lung, our group has pioneered human alveolar epithelial cell models, including primary cells and as cell lines. Complex human *in-vitro* models allow to mimic diseases like inflammation and infections by bacterial biofilms [11-13]. Novel self-assembling nanocarriers, capable to co-deliver Tobramycin and modern quorum sensing inhibitors, allow to significantly reduce the dose of the antibiotic for completely eradicating the bugs and thus also the risk of inducing antimicrobial resistance [14,15]. Apart from ethical advantages, predictive *in-vitro* models, ideally based on human cells and tissues, may be powerful tools to speed up the translation of novel anti-infectives and targeted delivery systems thereof into the clinic compared to often used animal models.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is no ethics vote applicable.

189

TPI.tv: A visual platform for animal-free innovations

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Knowledge on animal-free innovations is primarily shared via scientific papers: lengthy, decent pieces of text that extensively describe a small piece of knowledge. While this is crucial to move the science forward, this type of information is not easy to digest and is scattered in the scientific literature.

Especially in a field that is in transition, scientists need a platform to connect, exchange their ideas, get inspired. For this reason, we developed a video platform, TPI.tv. This platform can be thought of as a digital coffee corner where experts share new concepts, innovations and questions in an easy-to-access manner, via video. Video provides an attractive and inspiring medium that connects professionals working in the chain of animal-free innovations – from research and development through actual application by end-users – across borders and disciplines.

TPI.tv is an ad-free, curated website that currently contains over 80 videos [1]. These videos are 1-3 minutes long and serve as a gateway to more information on what is presented, connecting to publications, protocols, websites, data and social profiles. In this way, TPI.tv serves as a dissemination tool that facilitates dialogue on animal-free innovations. Through knowledge sharing, TPI.tv aims to enhance the development and application of animal-free innovations in basic science, regulatory science, translational science and education. TPI.tv is part of the Dutch transition program on animal-free innovation (TPI) [2].

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

190

AELVi "Arlo" – A novel monoclonal human alveolar epithelial cell line with reproducible barrier function

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The need for suitable models to mimic the human *in vivo* situation is constantly increasing in order to replace, refine or reduce animal experiments. Reducing the complexity of the *in vivo* situation requires that such models reproducibly reflect essential physiological functions *in vitro*. A model of the air-blood barrier for testing safety and efficacy of new pulmonary drugs and formulations has to provide a tight diffusional barrier, reflected by the high transepithelial electrical resistances (TEER).

The previously reported hAELVi cells (human alveolar epithelium lentivirus immortalized) develop electrically tight barrier properties with TEER values > 1000 Ohms*cm^2. They have been suggested as a promising cell type to replicate the human alveolar epithelium *in vitro* for biopharmaceutical applications [1,2]. The hAELVi cells are composed of a heterogeneous cell population since its initial immortalization was conducted concurrently on a mixed population of primary human alveolar epithelial cells (hAEpCs) *in vitro* [1]. The heterogeneity of the hAELVi cells, due to the batch-to-batch variance, led to differences in TEER value development, which may result in non-reproducible transport experiments. In addition, the hAELVi cells showed a multilayered morphology from as early as 7 days of *in vitro* culture, which also interferes with consistent results especially in case of molecular transport experiments.

We thus generated a single cell clone from the polyclonal hAELVi cells which was named "hAELVi Arlo" via single cell printing [3]. Arlo shows reproducible TEER value formation with values > 1500 Ohms*cm^2 under submerged as well as air-liquid interface (ALI) conditions, together with stable growth in a monolayer over a culture period of 14 days. Transcriptomic analysis performed on cells cultured on Transwell® inserts for 14 days under ALI-conditions further revealed that Arlo expresses genes relevant to barrier integrity and homeostasis that show striking similarity to genes expressed by hAEpCs, with only certain restrictions. Confocal imaging also indicated the presence of tight junctional complexes, demonstrated by the detection of tight junction protein 1 (TJP1/ZO-1) together with occludin (OCLN) that both showed continuously connected protein networks. Transport experiments additionally demonstrated functional barrier properties. Challenge with EDTA as tight junction modulator led to an increase of the apparent permeability of Fluorescein sodium together with a strong reversible decrease of TEER values. As the barrier properties of primary cells are still regarded as the gold standard in biopharmaceutical research [2], the high similarity of these properties and the expression pattern of Arlo compared to hAEpCs makes Arlo a more than promising tool to replicate the function of the alveolar epithelium *in vitro*. Furthermore, Arlo showed good performance in preliminary co-culture models, and inflammatory models.

Further characterization is planned to understand and achieve a better *in vivo*/*in vitro* correlation of barrier relevant function at cellular level including modulators like inflammation. In a first step mass spectrometric driven proteomic characterization of Arlo, upon *in vitro* barrier formation, will allow us to identify critical players in barrier function (e.g., expression patterns of Claudins) and their correspondence to the *in vivo* data available.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

191

A Bayesian network tool for predicting fish acute toxicity – Can simple behavior tests in fish embryos be used to increase confidence in predicted effect concentrations?

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The fish embryo toxicity test (FET; OECD TG 236) shows an overall very high, close to 1:1 correlation with the acute fish toxicity test (AFT; OECD TG 203). Therefore, in order to reduce the number of fish tests required for hazard assessments of chemicals it has been proposed as an alternative to the AFT. The European Chemical Agency (ECHA) has indicated that FET data can only be used as a replacement for AFT data as part of a Weight of Evidence (WoE) approach. The reason for the limited acceptance was the observation that a few compounds, such as neurotoxic chemicals, exhibited a weaker sensitivity in the FET. How a WoE approach could be used in practice could be challenging. Therefore, in order to strengthen the ability of FET data to predict (juvenile) AFT we developed a probabilistic approach using a Bayesian network (BN) model to support the use of alternative experimental approaches such as FET data. The model was developed as part of the CEFIC LRI ECO51 project SWiFT (Strengthening Weight of evidence for FET data to replace acute Fish Toxicity; https://swift.hugin.com/models/FET/). It uses various types of evidence including chemical structure, physicochemical data, daphnia and algae toxicity, gill cell-line cytotoxicity (ISO 21115, OECD 249), embryo toxicity and biotransformation. A curated training dataset was used to generate prior probabilities and to assign weights to the three lines of evidence based on existing experimental data. The existing high quality experimental toxicity data (LC50 and EC50 values) were combined into three main lines of evidence, while other model predictions (QSAR) were used to inform prior probability distributions. Additional supportive information on neurotoxicity and biotransformation was used to indicate the suitability of the BN model for the given substance. We present the model and approaches evaluating its performance and defining its applicability domain. We will demonstrate – given the concern with regards to neurotoxic mode of action – how information on (potential) neurotoxicity can be considered in probability predictions (e.g., via comparison of daphnia/algae toxicities). As another possibility to incorporate neurotoxicity information we proposed using simple behavior (movement) assessments in zebrafish embryos. While sophisticated behavior assessments using video tracking allow a more quantitative and unbiased assessment, we showed that simple microscopical observations may provide the opportunity to provide information on a potential neuroactive mode, demonstrated by the comparison of 10 neurotoxic and 4-non neurotoxic model compounds. The advantage of the simple assessment is that it can be conducted as part of the TG 236 without the need for further specialized equipment. At present the behavior information can only be used as a qualitative endpoint to support the confidence in the observations. With increasing data availability behavior endpoints could be incorporated into the probability predictions of the BN.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Hormonal influence on the primordial germ cell proliferation and distribution in the early development of the medaka

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In the gonochoristic medaka (*Oryzias latipes*), males and females show different numbers of primordial germ cells at hatching [1]. In females, estrogen mediates the type II proliferation, a faster concurrent division starting at developmental stage 38, resulting in a higher PGC number in genetic females. We created two transgenic lines for (i) the identification of PGCs and (ii) the identification of chromosomal sex by fluorescence microscopy. In the double transgenic offspring, we tested our hypothesis that endocrine disruption could affect the PGC number in in a Medaka embryo Reproductive Toxicity Assay (MeRTA). The sex hormones E2 and T initially seemed to lower the PGC count while the endocrine disruptor BPA showed no effect. However, we observed a sex-dependent difference in clustering vs. dispersed localization of the PGCs in the early gonad being indicative for the sex specific proliferation type of PGCs. The hormone E2 and the endocrine disruptor BPA increased the number of individuals with PGC clustering phenotype.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

193

A mouse pup model for learning the phalanx distalis amputation and pup handling

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In biomedical research involving animals, identification methods are crucial. By their use, results can be associated with individual animals. There is a number of techniques, applied by animal care technicians, as well as scientists. If animal identification is lost, results may become unviable. Further, certain techniques yield genotypable material, preventing additional procedures on an animal.

The Phalanx-Distalis-Amputation (PDA) is an invasive technique, used for mouse pups to be marked and genotyped at a young age. For that purpose, the most distal phalanx is removed with scissors. The procedure is best carried out on mouse pups of five to seven days of age. The removed phalanx can be used for genotyping [1].

As the removal is permanent, the animal can be identified over the course of its life. Complications arise when the nail bed is not completely removed leading to regrowth, or if more than the required distal phalanx is amputated. Therefore, an adequate education is required for the method. We developed a mouse pup model including the phalanxes in detail, so it can be used for teaching the PDA. By *ex vivo* µCT imaging we created a three-dimensional dataset, out of which we segmented and 3D-printed the pup model.

The model is currently used in the education at our institution, where we evaluated the pup model for ifs capacity to replace animals in teaching the technique. Preliminary results show the affinity of users to the prior experience the model offers for both handling pups and the PDA. Going forward, we will adjust the model to enable the practice of more techniques around animal identification and handling.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

194

Modeling the *in utero* electroporation and surgical techniques beyond for the replacement of animals in teaching

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The laboratory mouse is the most commonly used animal in biomedical research [1]. The adequate training of new personnel in animal care and scientific procedures is necessary for both obtaining optimal results, and for improving animal welfare according to the 3Rs after Russell and Burch.

Specifically surgical techniques, used in both basic and applied research, can be a challenge for new scientists to learn, resulting in large numbers of animals used exclusively for mastering the surgical method. With this project, we developed a framework for the production and creation of mouse surgical models, which can be used to replace animals in the learning and teaching of surgical methods.

As a primary use case, we applied the framework to the In-Utero-Electroporation (IUE). The IUE is a complex technique, where genetic material is injected into the lateral ventricle of the mouse embryonic brain [2]. By using a six-step process we were able to create a uterus and an embryo model out of soft silicones. Embryos and uteri were imaged by µCT. The three-dimensional datasets were processed into 3D-models and ultimately molds were created from the models and 3D-printed. With these molds we were able to cast realistic models, using silicone to mimic soft tissues. In a survey we conducted, users and trainees rated our models as realistic and a support in learning the IUE.

Beyond the IUE, the six-step process enables scientists to intercept at any step for the creation of new models. Thereby, one may create new scans to apply the whole process, work on digital models for new types of molds, or 3D-print and cast existing models for the application in the teaching of surgical methods. The used 3D-printer and silicone were applied to this process explicitly for being relatively cheap and affordable. Therefore, a large variety of labs will be able to use this process to adjust and create models, to replace animals in biomedical research.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Target group specific efforts to facilitate replacement of animal experimentation

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The Swedish 3Rs Center and its steering group, the Swedish National Committee for the Protection of Animals used for Scientific Purposes, works with all three Rs and with activities directed to many different target groups. This abstract describes our work specifically with the Replace R and how our efforts within this area are adapted to some of our dedicated target groups.

Government agencies, animal welfare bodies, researchers and students are examples of target groups to which the 3Rs Center directs its efforts. In some cases, the groups converge and can benefit from the same type of activities while in other cases specific materials or activities are needed.

With respect to government agencies, the 3Rs Center's efforts are focused on collaboration and dialogue. Each government agency that come into contact with animal experimentation, directly or indirectly, has its own specific needs to move towards animal-free methods. The 3Rs Center thus provide agency-specific lectures to increase knowledge about specific methods or concepts. The 3Rs Center also collaborates with government agencies to identify and finance Swedish experts for work in various expert groups with the aim to speed up the development of animal-free test guidelines.

With respect to researchers, the 3Rs Center works to increase the knowledge about animal-free methods and to facilitate networking. The Swedish 3Rs Network for Replace was instated in 2019 and today has more than 300 members. The 3Rs Center disseminates information in the network and also offers the possibility for members to start discussions and ask questions in an associated LinkedIn group. Representatives of government agencies, animal welfare bodies, research-funding bodies, etc. are also present in the network and thus these efforts converge on several target groups.

The 3Rs Center's efforts to facilitate the animal welfare bodies' work to contribute to Replace is a new initiative that started during 2022. In the upcoming years the 3Rs Center will meet animal welfare bodies in meetings and workshops to hear about their work, collect ideas and find out how the Replace-work can be facilitated. One aim is to eventually be able to organize local workshops to work with concrete research related questions and facilitate Replacement.

With respect to students, or more specifically the upper secondary school, the 3Rs Center has compiled digital education material focused on research, animal experimentation, ethics and animal-free methods. The lectures include information in both text, image and film and questions to discuss in the class. The aim of this material is to increase knowledge about research and different research methods already at an early age and open up to a neutral discussion without polarization.

The 3Rs Center is also working to compile a national strategy for replacement of animal experimentation. In this work we aim to include all target groups in order to achieve a successful strategy with a potential for positive change.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: No ethics vote is required.

196

Application of peptide reactivity assays for quantitative prediction of skin sensitization potency

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The Direct Peptide Reactivity Assay (DPRA) is a well-established test guideline method to assess whether a chemical is reactive enough to sensitize and is used together with *in vitro* testing in defined approaches to assess skin sensitization potential of chemicals on a yes/no basis. It involves incubating the test chemical at a single dose with a cysteine-based peptide for 24 h and analyzing for the depletion of peptide (DP). A lysine-based peptide can also be used in a parallel assay. If the DP value exceeds a specified threshold, the result is considered positive [1].

The Kinetic Direct Peptide Reactivity Assay (kDPRA) has recently been accepted as an OECD test guideline [1]. It is similar to the DPRA but uses a range of test chemical concentrations and a range of reaction times so as to produce a matrix of DP values. This data matrix is analyzed by a defined mathematical protocol to produce a reactivity parameter, $\log k_{max}$, which can be used as a standalone method to assign chemicals between the 1A and 1B potency classes [1].

Although both the DPRA and the kDPRA have their limitations [2], they have the potential to provide more than simply yes/no and 1A/1B information. In particular, as will be illustrated here: i) by modifying the kDPRA data matrix analysis, true rate constants can be derived and used in mechanism based QSARs to predict potency on a continuous basis, and ii) the presence of more reactive impurities can be detected, and their effects quantified. Evidence is found that one of the compounds in the kDPRA database that contains a more reactive impurity is the "standard contact sensitizer" 2,4-dinitrochlorobenzene (DNCB), probably the most extensively used in mechanistic studies over many decades. Fortuitously, this does not invalidate the many earlier findings based on data from experiments with DNCB.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

197

Use of assay ready THP-1 derived macrophages to test for pyrogen contamination

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Testing for pyrogen contamination is one of the most critical QC assays for revealing regulatory approval since this type of contamination can cause severe reactions in the human body even on low concentrations. Therefor highly sensitive assays are needed to predict the pyrogen potential of a product. Commonly the rabbit pyrogen test is used to determine the risk potential, but recently cell-based assays have also been accepted as alternative by pharmacopoeia, such as the MAT Assay. The monocyte activation test can measure the amount of pyrogen found in those products; it is therefore an efficient alternative to the undesirable rabbit pyrogen test.

We have tested THP-1 derived macrophages which were cryopreserved as assay ready cells, meaning prior cultivation is not necessary, these prequalified assay ready cells are more precise than a continuous culture due to lack of variances from cell handling and passaging. The assay ready cells function like a reagent in the monocyte activation test and were neither less sensitive, nor did they display a higher sensitivity when treated with endotoxins and non-endotoxin pyrogens compared to cells from a continuous culture.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

198

Use of assay ready KeratinoSens® cells to test for skin sensitization

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To evaluate the skin sensitizing potential of chemicals, reporter skin cell lines are used to measure the activation of the ARE/Nrf2 pathway, which is one of the key events of this complex cascade. Within the context of the keratinocyte activation, the KeratinoSens® cell line has been developed by Givaudan and validated by the ECCVAM.

The reproducibility of cell-based assays strongly depends on the cell quality, which in turn is influenced by multiple factors such as the choice of the culture media and sera, the source and passage number of the cell line, or even slight differences in cell handling by different operators. Thereby, all these parameters need to be optimally standardized. For this, the use of pre-made and pre-qualified Assay Ready Cells, which can be applied in a cellular assay basically like a reagent without prior cultivation or passaging, can minimize the variability related to cell culture.

Here we demonstrate that the use of this cell line in an assay ready format, to test the proficiency substances according to the OECD guideline 442D, leads to equivalent results as compared to continuously cultured cells. Assay Ready Cells were neither less sensitive, nor did they display a higher sensitivity. Basal level of Nrf-2 activation was not elevated in the assay ready cells, suggesting that the cells were not exposed to oxidative stress during cryopreservation from which they would need to recover after thawing.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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3D bioprinted, vascularized neuroblastoma microenvironment for studying tumor-angiogenesis and metastatic processes in fluidic chip devices

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Neuroblastoma is an extracranial solid tumor that develops in early childhood and still has a poor prognosis. One strategy to increase cure rates is the identification of patient-specific drug responses in tissue models that mimic the interaction between patient cancer cells and tumor environment. We developed a perfused tumor – microenvironment model that is directly bioprinted into laser-manufactured fluidic chip devices. A gelatin-methacrylate/fibrin-based bioink containing multiple cell types mimics the tumor-microenvironment and promotes spontaneous capillary formation by embedded endothelial cells. A specific design of the fluidic devices stably anchors the bioprinted tissue equivalent also for long-term perfusion in the device. Both, fluidic chip devices and bioprinted tissue can be fabricated at a medium throughput level. We demonstrate that adipocyte- as well as iPSC-derived mesenchymal stem cells can guide the capillary formation process. Bioprinted channels are coated with endothelial cells post printing to form a dense vessel – tissue barrier. The tissue model thereby mimics structure and function of human soft tissue with endothelial cell-coated larger vessels for perfusion and micro-vessel networks within the hydrogel-matrix. Patient-derived neuroblastoma spheroids are added to the matrix during the printing process and are grown for up to two weeks in the soft-tissue chip. We demonstrate that micro-vessels are attracted by and grow into the tumor spheroids and that neuroblastoma cells from the spheroid invade the tumor-environment along capillaries as soon as the spheroids disrupt. Thus, initial mechanisms of tumor angiogenesis and the first steps of metastasis can be directly assessed and visualized in this model. Since all necessary cell types for this model can be differentiated from iPSCs, the system will allow personalized drug testing and dose optimization in precision medicine applications. In summary, we developed the first 3D bioprinted, micro-vascularized neuroblastoma – tumor-environment model directly fabricated in fluidic chip devices and a novel medium-throughput biofabrication platform for parallelized anti-cancer drug testing.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The human 3D liver spheroid model is a pragmatic DILI hazard identification approach for early drug discovery phases

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Animal models are used since the 1960s as a "complete *in vivo* test system" for toxicity testing of new drug candidates. This practice is endorsed by regulatory authorities and therefore currently well-established in the pharmaceutical industry. About 30% of all drug candidates tested in preclinical animal models fail to reach first-in-human (FIH) studies due to induction of DILI (drug-induced liver injury). However, 90% of the drug candidates not found to be hepatotoxic in preclinical animal models nevertheless fail in FIH studies either due to the induction of clinical DILI or lack of efficacy or other liabilities [1]. This observation shows that animal experiments do not accurately predict the clinical hepatotoxicity of drugs and that DILI prediction must be further improved.

Complex micro physiological systems (MPS) raised expectations that their use in industrial practice would be a game changer in terms of better DILI prediction. However, only a few made it beyond proof-of-concept testing into the regular industrial application workflow once the evaluation of their predictive power and applicability were concluded.

In the following work, we introduce the 3D InSight™ liver MPS as a high-throughput (96- and 384-well-plate) standardized primary human cell-based *in vitro* model that recapitulates the essential structural and functional features of the native liver such as for example metabolic competency, cell polarization and bile acid synthesis. This MPS is a co-culture of human liver sinusoidal endothelial (LSEC), Kupffer cells (KC) and primary hepatocytes isolated from 10 donors (5 males and 5 females) to model an average of the population accurately. Previous work demonstrated that this MPS predicts clinical hepatotoxicity more accurately than 2D cell-based models [2,3]. The effect of 110 clinically DILI drugs on the cellular ATP level of the model was determined and IC_{50} values were ranked according to their hepatotoxic potential. The model predicted hepatotoxic drugs with a specificity of 83% and a sensitivity of 61%, this latter figure being two-fold higher than the corresponding one achieved by a state-of-the-art 2D hepatocyte model. The sensitivity of this approach (i.e., "DILI assay") is significantly further improved when its results incorporate human exposure data and benchmarked against the DILIrank, an FDA dataset consisting of clinical drugs classified according to their clinical hepatotoxicity potential as "Most-DILI-concern", "Less-DILI-concern" and "No-DILI-concern". The cellular ATP IC_{50} of 63 DILIrank drugs was compared to their corresponding human exposure and the hepatotoxic potential of "Most-DILI-concern" drugs was very accurately predicted with a sensitivity of 90.3% and a specificity of 81.3%.

The model system can also be used for clinically relevant mechanistic studies because of the high accuracy of the assay. This MPS can also be used in a later phase of drug development to determine complex DILI patterns with "multiomics" methods (e.g., transcriptomics, proteomics, lipidomic and bile acid profiling) and the testing of drugs by co-incubation with specific pathway modulators to create a link between specific pathways and toxic response.

In summary, the high sensitivity and specificity of the 3D human InSight™ liver model for the prediction of liver toxicity testing makes it a useful MPS tool for *in vitro* safety assessment. Together with its flexible, fast, and cost-effective use, it enables the generation of big high-quality hepatotoxicity datasets important for the drug development process. This leads to a high level of pharma-compatibility and facilitates accurate internal go/no-go decisions.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

201

Humanized 3D cell culture materials – The next generation of *in vitro* testing

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Data from Humane Society International reveal that every year more that 115 million animals are used in laboratories throughout the world, moreover the National Institute of Health (NIH) has noted that 90% of all drugs tested on animals fail in humans. Beyond the ethical concerns, the use of animal models results in an inefficient, long and expensive drug discovery process. Regarding *in vitro* tests, 2D cell cultures are a practical option for screening assays, still they do not adequately mimic the complex and 3D organized cell native microenvironment. For that reason, 3D models are being adopted as platforms for cell culture and in the drug discovery process. We have developed a technology to synthesize human based cell culture platforms that overcome major issues in drug development and tissue engineering by using ethically sourced human proteins [1,2] The proposed materials have tunable mechanical properties, being versatile platforms for cell culture and microtissue development that will increase the accuracy of *in vitro* studies by providing realistic microenvironments to cells. These novel materials are easily processed in multiple geometries and in microarrays amenable to "organ-on-a-chip" systems and for instrumentation used in high-throughput (HTS) that are a need for pharma companies. They can find also applications as bioinks for bioprinting and tissue engineering applications.

The human based materials were produced from human proteins with origin in the extra cellular matrix (ECM) from amniotic membrane (AM) and platelet lysates (PL) from blood plasma. Placenta was manually dissected and separated in the amnion and the chorion membrane. The peeled membranes were decellularized and processed in a mill. Platelet lysates (PLs) were prepared by exposing pooled PRP to a series of freeze-thaw cycles. The obtained ECM proteins and PLs were chemically modified using a methacrylation protocol well established in our group resulting in methacrylated PL (PLMA) and amniotic membrane (AMMA) [1,2]. Degree and local of modification were evaluated by mass spectrometry. The methacrylated proteins were processed in the form of a hydrogel upon irradiation with UV light. Their structural and mechanical properties were evaluated. Total protein content and the release of specific bioactive molecules was done using Micro BCA and ELISA assay respectively. Cell culture was performed using endothelial cells, stem cells and multiple cancer cell lines either dispersed or in the form of spheroids.

The mechanical properties of the developed hydrogels, showed to be easily adjusted by tuning degree of methacrylation and concentration of proteins in solution. Protein release profile of shows an overall sustained release. It is also important to refer that the hydrogels maintain their shape during the time release tests were performed. The synthesized gels have proven to support distinct human derived cell cultures. We have demonstrated the superior bioperformance of such hydrogels compared to classical materials, and their versatility for spheroid invasion, endothelial cell alignment and stem cell culture [3,4]. Cells could perform important biological processes such as growth, sprouting and migration, displaying a good cell-matrix interaction. Moreover patterned hydrogels were able to provide a support for aligned cell growth. The innovation potential of our materials is based on the fact that contain human biochemical cues, is a complete xeno-free solution (in the absence of animal derived serum) for human cell culture and easy to manipulate [5].

This is a novel platform technology that is easy to manipulate, cost effective, reproducible and robust that will accelerate research and drug discovery by providing more realistic results. The benefits of using this next-generation of cell culture materials are both, economic and social, being mainly related to the 1) achievement of more accurate and clinical translatable results, 2) faster drug screening and development processes, 3) reduction in animal-model using and costs associated to late-stage drug failures.

European Research Council Proof-of-Concept grant agreement ERC-2020-PoC-957585 for the project Amniogel. C. Custódio acknowledge the FCT for the individual contract 2020.01647.CEECIND. This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 & UIDP/50011/2020, financed by national funds through the Portuguese Foundation for Science and Technology/MCTES.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Using perinatal tissues as a sustainable source of xeno-free platforms for cell culture

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Recently, three-dimensional (3D) cell culture platforms have emerged as valuable tools with potential to increase the accuracy of *in vitro* studies and aid reducing or even replacing animal models [1-3]. Inspired by the extracellular matrix (ECM) that compose living tissues, these platforms have been designed to support the attachment and growth of cells, therefore providing reliable data on how they behave and respond to stimulus when within their natural environments. Despite the great advances made on the design of such materials, including the development of ECM-based hydrogels, recapitulate the complexity and function of native ECMs, while producing cost-effective and safe materials, remains a challenge.

Towards this goal, here we propose the use of perinatal tissues as a source of proteins to develop 3D cell culture platforms. Amniotic membrane (AM) is the innermost part of the fetal membrane which surrounds and protects the fetus during gestation. The potential of this tissue has long been recognized in tissue engineering and regenerative medicine applications due to its richness in ECM proteins and other important components, including growth factors and cytokines [4]. However, its use for cell culture applications remains quite under explored, probably due to the lack of adequate mechanical properties of the so far described AM-based hydrogels. Here, we report the development of a chemically modified AM-based hydrogel which can be cured upon light exposure and tuned according to the intended application [5].

Human AM's were isolated from human placentas, decellularized and solubilized to produce a solution rich in ECM structural proteins and growth factors, and free of cells. Amniotic membrane methacrylated (AMMA) was then produced by reacting the soluble AM with methacrylic anhydride in different ratios in order to obtain variable degrees of methacrylation. AMMA hydrogels were synthetized upon exposure to UV light and characterized in terms of their biochemical, mechanical, and biological properties.

Results showed that AMMA hydrogels were robust and have tunable mechanical properties, allowing the production of scaffolds with variable sizes, formats and topographical cues. Additionally, they were able to support viability and proliferation of human stem cells up to 7 days in culture. This work showcases the production of a fully-human 3D cell culture platform produced from a cost-effective and readily available perinatal tissue.

We acknowledge the funding from the European Research Council (ERC) for the project Amniogel (957585 ERC-2020-PoC) and the Portuguese Foundation for Science and Technology (FCT) for the project BEAT (PTDC/BTM-MAT/30869/2017). Catarina A. Custódio also acknowledges the FCT for the individual contract 2020.01647.CEECIND and Inês A. Deus for the doctoral grant 2021.05271.BD. This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 & UIDP/50011/2020, financed by national funds through the Portuguese Foundation for Science and Technology/MCTES.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Magnetic 3D bioprinting as a surrogate to aorta ring assay

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To meet the demand for better and higher throughput *in vitro* alternatives to animal models, we will present the results of a novel magnetic 3D bioprinting assay ideally suited to vasoactivity testing as a surrogate for *ex-vivo* aorta-ring assay.

Vasoactivity, or the contraction and dilation of blood vessels, is a key biomarker in evaluating and developing new drugs. For the pharmaceutical and biotechnology industries, adverse toxic events in the cardiovascular system, including vasoactive responses, account for the largest number of market withdrawals at 45% [1]. Vasoactivity can also be an endpoint for skin and tissue permeability redness, which is important to the cosmetics industry. Given this importance, evaluating the vasoactive responses before market entry is critical and avoids a costly market withdrawal.

However, while biomedical research moves towards high-throughput screening workflows to screen thousands of candidate compounds efficiently, no high-throughput *in vitro* assay exists to screen for vasoactivity. The standard assay for vasoactivity is wire myography, or the aortic ring assay, where the contractility of an *ex vivo* blood vessel is measured after exposure to a compound. As with most animal models, wire myography suffers from high costs, inflexible timing and processing, ethical challenges, low throughput, and variability between species that ultimately limits the predictive value of the assay [2,3]. Thus, there is an unmet need for a high-throughput *in vitro* assay to accurately screen for vasoactivity.

Moreover, the limited relevance of non-human tissues and the need to reduce the dependence on animal-intensive tests like wire myography define an unmet need for *in vitro* assays with fewer ethical challenges and the potential for greater translational capabilities. Thus, there is a demand for an *in vitro* assay that is predictive of *in vivo* vasoactive responses, uses human cells and is adaptable to high-throughput screening, as either an alternative to wire myography or earlier screen for vasoactivity before wire myography.

Here, we will present a valuable biomarker using real-time high-throughput imaging to phenotypically profile cell-cell and cell-drug interactions. This is achieved by combining the speed of magnetic 3D Bioprinting, rapid real-time imaging, and principle component analysis (PCA) to characterize and use the dynamic process of three-dimensional cell assembly as a biomarker of cell-drug interaction. The principle behind this assay is the magnetic 3D bioprinting of vascular smooth muscle cells into 3D rings or dots that structurally and functionally mimic blood vessel segments capable of contraction and dilation [4,5]. Also, a cost-effective imaging modality with a mobile device was developed to capture cell ring contraction in real-time in high-throughput. Rat (A10) and primary human vascular smooth muscle cells were magnetized with biocompatible nanoparticles (Nanoshuttle™), levitated for two hours, then rapidly magnetically bioprinted into 3D rings (toroid structures). Compounds with known vasoactive responses were studied where the contraction of the bioprinted rings was measured in real-time for 24 h. As expected, vasoconstrictors accelerated ring contraction, while vasodilators slowed it – these responses corresponded with changes in phosphorylated myosin light chain as seen by immunohistochemistry. Moreover, gene expression analysis suggests that cells within rings compensate genetically in response to vasoactive effects. This is a novel assay developed in 96- and 384-well formats where changes of 3D bioprinted rings is used as an endpoint for vasoactivity, addressing the need for high-throughput *in vitro* alternatives to wire myography to reduce and replace the use of animals.

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Submission declaration:
Conflicts of interest: The corresponding author declares that he receives royalty payments from the University of Texas MD Anderson Cancer Center, Greiner Bio-One, and Rice University as one of the inventors of this technique.
Statement on ethics vote: There is a positive ethics vote.

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Human placental villous tissue explants as basis for 14-day toxicity studies

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Numerous forms of placentation have evolved over the course of evolution, with species differing greatly in terms of anatomy, endocrinology, and immunology. Therefore, an animal model that completely reflects human placentation does not exist [1,2]. On the other hand, the human placenta is an organ permanently available for research in a healthy state, which offers the opportunity for various *ex vivo* approaches.

One of these approaches is the use of placental villous tissue explants. Such explants, prepared from the placenta after birth, can be cultured for up to several weeks and provide a promising basis for toxicity studies as well as basic research. Once in culture, placental explants undergo dynamic development. As monitored by estradiol/progesterone production and observed microscopically, the original syncytiotrophoblast initially degrades but is then replaced by a new one. We consider this process, which involves proliferation and syncytialization, to be a promising target for toxicology. In 14-day cultures we proved, that it is possible to disrupt explant regeneration by exposure to toxicants and showed that the experimental setup can be improved by using human serum instead of fetal bovine serum.

An interesting further development is the dynamic culture of placental villous tissue explants in bioreactors under superfusion, with the aim of better reflecting the *in vivo* situation. Placental explants are sensitive tissue structures that lose their integrity under fluid shear stress. We examined the fluid stress effects on explants under different fluidic regimes. Following the observation that placental explants are very sensitive, we designed a new scaffold structure that combined the potential shelter function of a porous cavity with the advantage of better fluidic supply with respect to nutritious flow. The scaffold called TissGrid® enables excellent regeneration of the placental explant [3].

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Modernizing medical research to benefit people and animals

Presented on behalf of Isobel Hutchinson, Carla Owen and Jarrod Bailey
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The use of animals in research has been the subject of significant public debate and concern. Some argue that animal experiments are essential to medical progress, while others believe that this practice is unethical and does not produce results that can be reliably translated to people. A growing range of modern techniques can replace the use of animals and provide results that are more relevant to human patients. These include the use of human cells and tissues, and computer-based methods. Our work aimed to explore the societal benefits of accelerating the replacement of animals with these new research methods.

There is a powerful case for modernizing medical research by replacing animals with human relevant techniques. This approach will provide the best possible chance of accelerating progress in tackling the major public health challenges that have a terrible impact on patients and place strain on public healthcare systems, providing better results for people, more quickly. This transformation would help to spare animals from being subjected to experiments that inherently involve suffering. Engaging with the New Approach Methodologies (NAMs) sector also provides significant economic opportunities. Within a British context, transforming research in this way would align with the public and policymakers' priorities and help to place Britain at the forefront of the global shift towards animal-free research. The evidence in favor of modernizing medical research is becoming increasingly difficult to ignore, and policymakers should embrace this more ethical and future-focused approach to research.

We want to discuss and empathize the essential role of governments, and how they can set out key recommendations for policymakers. In May 2022, Animal Free Research UK published a review covering this topic [1] and was hosted at the parliament to launch "8 concrete steps to accelerate human relevant innovation", sharing a plan mapping how cutting-edge non-animal approaches will be rolled out. This is an example of action that can raise policy makers (like MPs) awareness, to change mindset and enable changes in the law. All outcomes and ongoing work on that topic will be discussed.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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How to address animal methods bias in scientific publishing

Presented on behalf of Catharine E. Krebs[2], Helder Constantino[3], Lilas Courtot[1], Kathrin Herrmann[4], Ann Lam[2], Sofia Batista Leite[5], Janine McCarthy[2], Brinda Poojary[6] and Kristie Sullivan[2] by Lilas Courtot

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Publishing plays a crucial role in the advancement of science, the implementation of interventions, and the progress of researchers' careers. Thus, there is a need to identify and address biases that occur within the publishing process. We recently identified a bias called "animal methods bias in publishing:" a preference for animal-based methods where they may not be necessary or where nonanimal-based methods may be suitable, which affects the likelihood of a manuscript being accepted for publication [1].

On April 20 and 21, 2022, stakeholders gathered virtually for a workshop to address animal methods bias in scientific publishing. The charge for the workshop was: (1) Explore a range of stakeholder perspectives, including from academic and industry researchers, journal editors, and government representatives; (2) Describe the current state of animal- and nonanimal-based experimental systems; (3) Describe animal methods bias in publishing and related biases in publishing and peer review; (4) Identify potential causes, consequences, and mitigation strategies for animal methods bias in publishing.

Common and salient themes of the workshop were: (1) Different research domains have different needs for model systems and have made progress in nonanimal models at different rates; (2) Animal methods bias extends beyond publishing; (3) There is a need for more evidence of bias. Additional recommendations to address animal methods bias in scientific publishing that emerged from the workshop included: Build awareness; Educate editors and reviewers; Implement different incentive models; Require preregistration and retrospective review; Implement open science; Prioritize funding for nonanimal methods; Advance validation, standardization, and reporting of nonanimal methods; Change regulatory requirements.

This workshop was successful in its charge and created an ongoing taskforce committed to addressing animal methods bias in publishing, which will build more evidence and further explore contributing factors, impacts, and mitigation strategies. As a first step in the process of building more evidence, the steering committee is currently working on a report of the workshop that will be submitted soon for publication.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Fostering awareness, accessibility, and acceptance of animal-free antibodies

Presented on behalf of Niamh Haslett[2], Michael Cook[2], Jesus Calvo-Castro[2], Stewart Kirton[2] and Jarrod Bailey[1] by Lilas Courtot[1]

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There is a general lack of awareness around the topic of animal-free antibodies for research. Their status of being relatively "new" raises questions around their validity and quality. Many suppliers and researchers are hesitant to change materials or techniques that are well established without due cause. Some don't not know they are even an option available to them. Currently, most antibodies used in research are derived from animals, with about 1 million animals used in their production each year in Europe. New animal-free technologies are emerging, with techniques like phage display gaining worldwide recognition as the way forward for antibodies, as more publications are reporting improvements in their research due to using animal-free antibodies.

An initial project of an online database with an accompanying informational website is in development to facilitate the procurement of animal-free antibodies. It also aims to serve as a visualization tool for antibody suppliers to gauge increasing demand levels for such products. A review was conducted to establish current mainstream antibody production methods and evaluate the new approach methodologies used to introduce animal-free antibody products to the market. The advantages and barriers to their widespread use were outlined and used as justification for the critical design of an online resource. A dedicated steering group has provided integral advice and support from their experienced positions at the top of their fields in order to cater to every target audience.

Traditional methods of antibody production utilize animals such as rabbits, goats, and sheep, in at least one step of their process, primarily acting as hosts for antibody generation. Animals can be subjected to continuous cycles of antigen exposure via injection and blood extraction for polyclonal antibody production. For monoclonal antibody production using hybridoma technology, mice are unreservedly sacrificed for the retrieval of spleen cells. Alternatives are continuously being explored, with newly developed methods providing feasible production of recombinant monoclonal antibodies with zero animal involvement and associated publications indicating the final products outperform their animal-derived counterparts. Phage display uses bacteriophages encoded with human antibody DNA to generate monoclonal antibodies on their surfaces. More "animal free" examples exist, including other display techniques, computer-assisted 3D modelling, and synthetic antibodies generated with Molecularly Imprinted Polymers (MIPs).

The emergence of these technologies can allow improved reproducibility for research, diagnostic, and therapeutic purposes, as well as having potential for greater functionality and upscale without animal use. The European Centre for the Validation of Alternative Methods [1] (ECVAM) recommended the induction of a major phase out of animal derived antibodies, as well as tightening criteria for publications involving their use. As part of this initiative, Animal Free Research UK and the University of Hertfordshire are collaborating to create interactive resources and online tools to assist researchers in their transition to practicing animal-free science wherever possible.

The limitations of currently accepted antibody generation methods are highlighted by showcasing the advantages of modern antibody production techniques with potential to be animal-free. This is the basis of the justification for creating an informative, collaborative online resource to be utilized as an initial step in the procurement processes for scientists undertaking antibody research.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

208

The future of Parkinson's disease research: A new paradigm of human-specific investigation is necessary

Presented on behalf of Manuela Cassotta[2], Hugo Geerts[3], Lise Harbom[4], Tiago F. Outeiro[5], Iosif Pediaditakis[6], Orly Reiner[7], Stefan Schildknecht[8], Jens C. Schwamborn[9], Jarrod Bailey[1], Kathrin Herrmann[10,11] and Helena T. Hogberg[10]
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Parkinson's disease (PD) is a complex neurodegenerative condition with a multifactorial origin. To date, approaches to drug discovery for PD have resulted in symptomatic therapies for the motor manifestations and signs associated with neurodegeneration but have failed to identify preventive or curative therapies. This failure mainly originates from the persistence of major gaps in our understanding of the specific molecular basis of PD initiation and progression. New approach methodologies (NAMs) hold the potential to advance PD research while facilitating a move away from animal-based research.

In 2021, the Center for Alternatives to Animal Testing (CAAT) and the Center for Contemporary Sciences (CCS) coordinated and held a workshop to develop a scheme that outlines the basic research and testing approaches necessary to make PD research a success. This workshop involved NAM experts in the field of PD and neurodegenerative diseases, who discussed and identified a scientific strategy for successful, human-specific PD research elucidating pathological mechanisms, identifying knowledge gaps, discovering druggable targets, and developing new therapies that can enter the drug development pipeline – focusing on human-specific, non-animal methods of research. They outlined some of the most important human specific NAMs, along with their main potentials and limitations, and suggest possible ways to overcome the latter [1].

Key recommendations to advance PD research were discussed, including integrating NAMs while accounting for multiple levels of complexity, from molecular to population level; learning from recent advances in Alzheimer's disease research; increasing the sharing of data; promoting innovative pilot studies on disease pathogenesis; and accessing philanthropic funding to enable studies using novel approaches. Collaborative efforts between different stakeholders including researchers, clinicians and funding agencies are urgently needed to create a scientific roadmap and support a paradigm change towards effective, human-specific research for neurodegenerative diseases without animals, as is already happening in the field of toxicology.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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New non-invasive, label-free monitoring approach for high cell culture and assay quality

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Monitoring cell viability under culturing conditions provides a measure to address the principles of GCCP [1]. Lensless Microscopy (LM) is ideal for this purpose since it allows cost-effective label-free live-cell imaging with compact instrumentation. We present a novel LM exploiting the optical properties of the cell itself as the imaging element or lens of the microscope. Cutting edge AI algorithms allow for fully automating the entire workflow from time-lapse imaging to proliferation and motility output parameters, leading to standardized protocols and constant conditions independent of the operator. We found that heterogeneous cell culture conditions lead to an increase of variance during subsequent assays like omics-readouts [2]. Enhancing the quality of cell culture ensures more reliable and reproducible results of subsequent experiments like, e.g., omics readouts, EC50 value determination, or treatment response.

The approach of time-dependent multiparameter monitoring in real-time inside the incubator including the determination of key cell culture parameters including confluence, proliferation, and clustering as well as cell migration [3] further allows for standardization and hereby enhancement of the quality and reproducibility of different cell-based assays, like wound healing assays, motility and proliferation assays, or spheroid growth monitoring. Continuous monitoring enhances the significance of toxicological and drug response assays compared to endpoint assays. Enabling faster and more reliable results of *in vitro* cell culture models, our approach contributes to the field of new approach methodologies in animal testing.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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The challenges and successes of promoting 3Rs in teaching and research in Italy: A Centro 3R update

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The Italian Centro 3R is an interuniversity center dedicated to promoting the 3Rs principles in teaching and research. It is the only center of its kind in Europe. Established in 2017, it boasts 8 universities and one research institute (INRIM: the Italian Institute for Metrological Research).

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 an important way of building knowledge on unanswered biological questions, it seeks to sensitize researchers to humane methods and humane ways of approaching science. 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.

Since our last report at EUSAAT, the Center has grown to over 500 members, with active participation in the implementation of teaching activities at all levels – undergraduate, postgraduate including masters and PhDs.

The Centro 3R has also taken an active role in training of professionals who deal with animal research, in accordance with national legislation.

The presentation will focus on the most recent efforts, including a workshop on new legislation in cosmetic testing and the REACH – EU directive 2010/63 conflict, new courses at the different universities. We will also discuss biomedical research being undertaken across Italy and the challenges faced by scientists in the light of what might be considered the excessive restrictions posed by the Italian translation of the EU directive.

Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote

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Puromycin aminonucleoside induces kidney injury in iPSC-derived kidney organoids

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Organ transplantation and kidney dialysis remain the only effective therapeutic tools against rising numbers of patients suffering from kidney diseases including acute kidney injury (AKI) and chronic kidney disease (CKD), which can progress to end stage renal disease (ESRD). For this reason, studying kidney diseases as well as the development of prospective therapeutic options and medications are urgently needed.

The kidneys are essential for bodily functions such as blood filtration and waste excretion, with the glomeruli and the renal tubules taking a major role. However, they are easily affected by changing cellular conditions leading to pathological conditions. *In vitro* models such as three-dimensional organoids represent a way to understand the mechanisms of kidney diseases and potential hubs for therapies [1]. Since they mimic the human anatomy and exhibit the physiological functions to an extent, organoid models are a promising alternative to conventional animal models.

In our study [2], we generated kidney organoids from three iPSC lines [3] derived from urine stem cells and treated them with puromycin aminonucleoside (PAN), which is a nephrotoxin and can be employed for inducing kidney injury. Our kidney organoids exhibited expression of kidney-related genes and morphological structures resembling renal glomeruli and tubuli. Moreover, a dextran uptake assay demonstrated the functionality of the tubular compartment. Glomerular and tubular structures within the kidney organoids displayed morphological disruption upon PAN treatment and additionally confirmed by transcriptome analyses. Subsequent analyses revealed an upregulation of immune response as well as inflammatory and cell death-related processes.

We conclude that treatment of iPSC-derived kidney organoids with PAN induces kidney injury mediated by an intertwined network of inflammation, cytoskeletal re-arrangement, DNA damage, apoptosis and cell death. In summary, we could demonstrate that iPSC-derived kidney organoids treated with toxic substances can be used as *in vitro* model for studying of kidney-associated diseases and drug discovery, and thus minimizing the use of animal models.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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A promising method for the determination of cell viability: The membrane potential cell viability assay

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Determining the viability of cells is fraught with many uncertainties. It is often difficult to determine whether a cell is still alive, approaching the point of no return, or dead. Today, there are many methods for determining cell viability. Most rely on an indirect determination of cell death (metabolism, molecular transport, and leakage, to name a few) [1].

In contrast, we have developed a promising novel method for a "direct" determination of cell viability. The potential method assesses cell membrane integrity (which is essential for all viable cells) [2] by measuring the electrical potential of the cell membrane. The electrical potential was measured with a commercial dye, FluoVolt™ (FV) [3,4]. To test the assay, we chose two different cell types, blood macrophages (TLT) and breast cancer epithelial cells (MCF 7). We exposed them to seven different toxic scenarios (arsenic (V), UV light, hydrogen peroxide, nutrient starvation, Tetrabromobisphenol A, fatty acids, and 5-fluorouracil) to induce different cell death pathways. Cells were observed for a period of up to 80 hours to establish a relationship between cell viability and FV intensity. We also observed the relationship between FV and cell viability using an imaging flow cytometer.

We observed a meaningful correlation between cell viability and the intensity of FV and developed a protocol for determining cell viability using the dye FV. Under controlled test conditions, the assay showed good accuracy when comparing the toxicity assessment with well-established methods. Moreover, the method showed compatibility with live cell imaging, to such an extent that stained cells were capable of mitosis at the same rate as unstained cells. Although we know that further studies are needed to confirm the performance of the assay in other situations, the results obtained are promising for their wider application in the future.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Expression dynamics of pregnane X receptor-controlled genes in 3D primary human hepatocyte spheroids

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The pregnane X receptor (PXR) is a crucial ligand-modulated transcription factor in liver being involved in regulation of both endogenous and exogenous metabolism. Up to date, a plethora of PXR ligands has been identified including rifampicin, which is often used as a prototype direct PXR activator in experimental studies. Although numerous PXR target genes were discovered, there is only limited knowledge on the temporal dynamics of their expression.

3D spheroids of primary human hepatocytes (PHHs) have emerged as a potential new gold standard of *in vitro* liver cell models. As compared with conventional 2D monolayer cultures, PHHs in 3D setting show relevant and stable hepatic phenotype.

In our study, we evaluated expression profiles of representative PXR target genes upon rifampicin treatment in a time course ranging from 4-168 h. The time period enabled to encompass early and late responses. We employed 3D PHHs, which were generated onto ULA plates and took advantage of their gene expression stability, a prerequisite for successful time expression profiling.

We found out a bell-shaped expression patterns for genes involved in xenobiotic metabolism (CYP3A4, CYP2C9, CYP2B6, and MDR1), which propose a coordinate program of those genes against xenobiotic challenge. Additionally, we determined half-lives of CYP3A4 and CYP2C9 mRNA as well as used mathematical model fitted to our *in vitro* CYP3A4 expression data to describe temporal dynamics of PXR-governed CYP3A4 mRNA induction in 3D PHHs. Moreover, we observed biphasic profiles for endogenous metabolism genes (FASN, GLUT2, G6PC, PCK1, and CYP7A1); downregulation for SHP or oscillation for PDK4 and PXR.

Overall, our results emphasize the importance of time expression profiling as one point or short-term analysis may lead to misleading conclusions in terms of a physiological impact of activated PXR. Moreover, an exceptional invariable phenotype of 3D PHHs provides insight into expression changes beyond limits of conventional 2D PHH cultures [1].

This study was supported by EFSA-CDN (No. CZ.02.1.01/0.0/0.0/16_019/0000841, Czech Republic), co-funded by ERDF.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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An overview of guidelines for better preclinical research

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Concerns about the quality of preclinical research are not new. Historically, these concerns focused on the harm inflicted upon animals, and they were raised by opponents of animal experimentation. More recently, scientists themselves have become increasingly critical of the validity, reproducibility and translatability of animal studies, challenging some of the central principles of traditional laboratory animal science. While this has occurred, the number of potential alternatives to animal use has risen rapidly. These events have resulted in a flood of information on potential ways of improving preclinical research, to which scientists are now exposed not only through the traditional scientific literature but also via social media. Scientists are facing legal, ethical and scientific demands to improve their preclinical methodologies.

Many established scientists have spent considerable time adopting, developing and refining animal models for use in preclinical research. Replacement of these methods by non-animal methodologies may be challenging and may be perceived as a threat to methods which they know from experience can be published. They may also have genuine concerns that "alternatives" do not answer the same questions as an animal experiment would do. Furthermore, they may be a temptation on both sides to "oversell" the value of their preferred method, whether it be an animal model or an alternative. There is, therefore, an urgent need for two types of guidance: how to improve preclinical research which still involves animal use, and how to find impartial information on the alternatives which are under development and validation.

Scientists who carry out animal research are usually well aware of reporting guidelines, such as ARRIVE [1], since these are often endorsed by the journals where they hope to publish, but they may be less aware of guidelines for *planning* preclinical research. Studies cannot be improved by a better description after they have been carried out. Planning and reporting guidelines are therefore both essential parts of the pathway to better research, at opposite ends of this process.

Norecopa's staff have spent many years producing, collecting and disseminating guidelines and practical tools for implementing all the three Rs. These are available in a free database, currently consisting of 8,900 pages, which is updated many times a week [2].

We have also developed a set of guidelines called PREPARE to encourage scientists to consider all three Rs from the earliest stages of planning preclinical research [3-4]. PREPARE is the result of discussions over many years with participants on courses in Laboratory Animal Science and with staff at accredited research animal facilities. PREPARE consists of a two-page checklist, available in over 30 languages, and a comprehensive website with links to resources to implement each topic on the checklist.

To further encourage the uptake of refinements to animal research, we have also created a Refinement Wiki, which can be used by scientists and animal care staff to publish improvements to the housing, care and use of research animals [5]. The aim of both the Wiki and PREPARE is to provide both scientists and animal facilities with resources to reduce the harm caused by animal research, either by employing alternative methods or by refining protocols which still involve animals.

This is a win-win situation: for science and for animal welfare.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

215

Proteomics reveals mechanisms of metabolic disruptive effects of emerging di-(2-ethylhexyl) phthalate substitutes

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The obesity pandemic and associated cardiovascular complications are presumed to be accelerated by endocrine disruptors such as the phthalate plasticizer di-(2-ethylhexyl)-phthalate (DEHP). However, the mode of action underlying their metabolic disruptive effects is insufficiently understood, and poorly studied emerging plasticizer alternatives require further investigation.

Given that adipose tissue functionality was reported to be impaired by phthalate plasticizers, we focused on the master regulator of adipogenesis, the peroxisome proliferator-activated receptor gamma (PPARγ) and examined 20 alternative plasticizers as well as their metabolites for binding to and activation of PPARγ and lipid accumulation in human preadipocytes. Among several compounds that showed molecular interaction with PPARγ, the metabolites MINCH, MHINP, and OH-MPHP of the plasticizers DINCH, DINP, and DPHP exerted the most potent induction of lipid accumulation [1].

These compounds were further analyzed in human preadipocytes and mature adipocytes using *in vitro* assays and global proteomics. In preadipocytes, the plasticizer metabolites significantly increased lipid accumulation, induced adipogenesis, enhanced leptin and adipsin secretion, and upregulated markers and pathways associated with PPARγ activation in a similar pattern to the PPARγ agonist rosiglitazone. Proteomics of mature adipocytes revealed that both, the plasticizers and their metabolites, induced a hypertrophic remodeling caused by oxidative stress and excessive deposition of extracellular matrix. This cellular stress led to impaired metabolic homeostasis, disturbed lipid storage, and induction of proinflammatory pathways as well as insulin resistance promoting adipokine secretion.

In conclusion, the plasticizer metabolites enhanced preadipocyte differentiation, at least in part through PPARγ activation and, together with their parent plasticizers, impaired the functionality of mature adipocytes similar to reported effects of a high-fat diet. This mechanistic knowledge may support adverse outcome pathway (AOP) development for the effects of xenobiotics on cardiovascular disease as investigated by the EU Horizon 2020 research project ALTERNATIVE. Moreover, this highlights (A) the need to further investigate the currently used plasticizer alternatives for potential associations with obesity and cardiovascular diseases and (B) the convenience of omics for regulatory risk assessment.

This work was supported by the German Federal Environmental Foundation (DBU). This conference presentation was supported by the European Union's Horizon 2020 research and innovation program (grant #101037090). The content of this manuscript reflects only the author's view, and the Commission is not responsible for any use that may be made of the information it contains.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.

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Ex vivo platform for feeding of ticks

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Ticks are obligatory blood feeders and notorious vectors of human pathogens. During blood feeding, ticks penetrate their mouthparts into a host's skin, and unlike other blood-feeders, are associated with the host circulatory system for up to ten days. This requires a very unique skill of secretion of an "invisible" potion to neutralize any host defense mechanisms, including itching, blood clotting, or swelling, which eventually also allows pathogen transmission. Tick research has been limited by the use of laboratory animals on which ticks feed.

Clear limitations include:

(i) tick saliva cannot be collected during feeding on live animals
(ii) the host blood composition cannot be manipulated in live animals
(iii) it is difficult to control levels of multiple compounds/cells supplemented to live animals (during tick feeding)

Ex vivo platforms address these concerns. Using this approach, we could follow up on genomic level data, indicating loss of haem biosynthetic pathway in ticks, and determine the nutritional dependence of ticks on host haem, by a comparative serum- vs blood-feeding assays [1]. Using small molecule inhibitors, which can be micro-injected into tick bodies, we can clearly determine, which proteins play a key role at the tick-host interface (being spit out with tick saliva) and filter out those that participate in the "house-keeping" physiology of ticks by demonstrating loss of phenotype when ticks are fed *ex vivo* [2] (i.e., where no host haemostatic or immune factors are present). This platform also allows oral toxicity assays of novel acaricides, which clearly surpass those based on laboratory animals in both ethics and economy.

Most current tick *ex vivo* platforms are based on a static system that requires a 12-h exchange interval for up to 10 days, making it a fairly laborious system. Fluidic approaches offer a systems-level understanding of biological problems. While the approach has been successfully applied across various scales (nano-, micro-) to produce cells- and tissues-on-a- chip, very rarely has it been applied to organism-scale research. Currently, we are developing a "tick-on-a-chip" platform to power novel insight into tick biology, inherently linked to their blood-feeding. Ultimately, we would like to devise a tracking system, likely based on Digital Holographic Microscopy that would allow a real-time detection and quantification of emitted pathogens in the outlet blood meal tube.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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A xeno-free *in vitro* 3D synovial membrane model mimicking the pathogenesis of arthritis

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Osteoarthritis (OA) and rheumatoid arthritis (RA) are the most prevalent forms of inflammatory joint diseases, resulting in cartilage degradation. Fibroblast-like synoviocytes (FLSs) of the synovial membrane have been identified as critical drivers of arthritis. However, knowledge of the impact of FLSs in the early stages of cartilage degradation is still limited. The pathogenesis of these diseases is studied primarily in animal models, which also represent an integral part of the preclinical drug discovery process. Although these animal models have limited predictive value and are accompanied by severe suffering for laboratory animals, they are still the gold standard due to the lack of complex *in vitro* alternatives [1]. Most *in vitro* models of the synovial membrane are based on RA-FLS or OA-FLS and animal-derived Matrigel™, which does not reflect the "healthy" state. Therefore, our ultimate goal is to develop a xeno-free, human-centered, *in vitro* 3D model of the synovial membrane as an alternative to animal models in line with the 3R principles.

To this end, we compared "healthy" FLS from trauma patients (trauma-FLS) to those derived from OA patients (OA-FLS) and bone marrow-derived mesenchymal stromal cells (MSCs) to identify a suitable cell source that mimics the synovial membrane *in vitro* most closely. The most suitable cell-type was incorporated into a synthetic RGD hydrogel (TheWell Bioscience Inc.) to generate a xeno-free *in vitro* 3D synovial membrane model. Finally, we confirmed the applicability of our model using cytokines involved in the pathogenesis of RA (e.g., 100 ng/mL TNF-α) and OA (e.g., 10 ng/mL TGF-ß).

Comparing the three cell types with regard to (i) minimal criteria that define MSCs such as plastic adherence, morphology, multipotency, and the expression/absence of surface markers, and (ii) fibroblast-related marker genes such as hyaluronan synthase 2 (*HAS2*), collagen type 3 alpha 1 (*COL3A1*), vimentin (*VIM*), and decorin (*DCN*) we could not distinguish the three cell types. Moreover, protein expression of HAS2, COL3, and VIM confirmed these findings. Thus, MSCs were used as a cell source reflecting the synovial membrane's physiological state. Next, we developed a synovial membrane model consisting of a lining and sublining layer by distributing MSCs into two hydrogel layers cultured in DMEM containing 2% human AB serum. Vertical histological sections confirmed homogeneous cell distribution, while cell viability for up to 10 days and biocompatibility of the hydrogel was verified by LIVE/DEAD™ staining, ApoTox-Glo™, and LDH assay. For future purposes, we confirmed the immunocompatibility of the hydrogel and its capability to facilitate immune cell migration. Treatment of our model with TNF-α to mimic RA increased MSC-proliferation reflecting the synovial pannus formation typical for RA. In contrast, OA-like treatment with TGF-β for three days already induced a fibrotic phenotype as reflected by increased protein expression analyzed by alpha-smooth muscle actin (α-SMA) staining on cross-sections compared to the untreated control.

In summary, the results of our study indicate that our xeno-free human *in vitro* 3D synovial membrane model is capable of mimicking cytokine-driven cellular changes as observed during arthritis. Prospectively, we aim to provide a complex *in vitro* alternative for preclinical drug screening following the 3R principles by incorporating monocyte-derived macrophages.

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Submission declaration:
Conflicts of interest: The corresponding author declares no conflict of interest with the authors.
Statement on ethics vote: There is a positive ethics vote.

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Fluidic shear stress reduces TNFα-mediated cartilage damage in a 3D model of degenerative joint disease

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Pathomechanisms of degenerative joint diseases such as osteoarthritis (OA) ultimately result in the breakdown of cartilage tissue. The exact underlying mechanisms of both cause and progression of OA remain fairly unclear. Even animal models, which are still the gold standard in OA research, do not provide sufficient information on human pathogenesis. Therefore, developing complex and long-lasting *in vitro* components of a human joint, including cartilage, subchondral bone, synovial membrane, and tendons that simulate the 3D architecture and the metabolic, humoral, and cellular interplay of the joint components, are needed to study the long-lasting course of OA pathogenesis. Besides the impact of metabolic features and 3D architecture, mechanical forces are well-known as essential modulators of joint health. In contrast, aberrant forces are primary etiological factors leading to cartilage degeneration.

Following the 3R principles, we aimed to (i) develop a long-lasting human xeno-free *in vitro* 3D cartilage model using alternated perfused cultivation and (ii) simulate TNFα-mediated cartilage degradation. We used perfusion-mediated fluid shear stress (FSS) to enhance chondrogenesis and mimic FSS during joint movement as a mechanical force.

Human bone marrow-derived mesenchymal stromal cells (MSC) were used to develop a xeno-free *in vitro* 3D cartilage model. These models were maintained in a bioreactor platform with a perfusion cycle that facilitates mechanical stimulation via FSS and daily sampling. Within the bioreactor, MSC mass cultures were subjected to FSS at 10 dyn/cm^2 by medium circulation three times a day for 1.5 hours. We compared the perfused approach using an optimized FSS rate, cycles, and cultivation period of 18 days to a non-perfused control. To this end, we measured cell viability (live-dead- and viability-assay), apoptosis (TUNEL-assay, caspase-3/7-activity, *BCL2/BAX*), metabolic activity (oxygen and glucose consumption, lactate production), chondrogenic gene expression (*ACAN, COMP, COL2A1, COL1A1, COL2A1/COL1A1*) and matrix metalloproteinase expression (*MMP-1, -3, -13*).

Alternate perfused long-term cultivation at 10 dyn/cm^2 did not affect cell survival; instead, it reduced apoptosis compared to the non-perfused control. Moreover, it did not affect oxygen consumption but reduced glucose consumption and lactate production and enhanced chondrogenic gene expression with reduced *MMP13* and *COMP* gene expression compared to the non-perfused control. Mimicking the pathophysiology of OA, we stimulated the 3D cartilage model with 100 ng/mL TNFα for 6 hours under non-perfused and perfused long-term cultivation with FSS at 10 dyn/cm^2 as a mechanical stimulus. Compared to untreated perfused conditions, TNFα stimulation (i) did not affect overall cell survival, but enhanced apoptosis (demonstrating efficacy of stimulation), (ii) did not affect oxygen consumption and glycolysis, and (iii) enhanced *COMP* and *MMP1* expression as markers of matrix protein turnover. In comparison to TNFα treated cells under non-perfused conditions, TNF stimulation under perfused conditions (i) did not affect cell survival but reduced apoptosis, (ii) did not affect oxygen consumption, but reduced glucose consumption and lactate production as a measure of glycolysis, and (iii) reduced the expression of *IL6* and soluble amounts of IL-6 but not of *TNFA* whereas soluble amounts of TNFα were enhanced. Furthermore, TNFα stimulation (iv) reduced the expression of matrix-degrading enzymes but (v) enhanced anabolic chondrogenic matrix proteins on mRNA.

In summary, in a xeno-free 3D cartilage model that mimics OA, FSS as a mechanical stimulus provides a metabolic "feel-good" niche that reduces chondrocyte apoptosis and metabolic activity, and matrix metalloproteinase expression increases matrix protein expression and protects against TNFα-mediated cartilage degradation. This approach convincingly demonstrates the feasibility of studying OA human-centered as an alternative to animal models.

Submission declaration:
Disclosure of interest: Thomas Leeuw is a Sanofi employee and may hold shares and stock options in the company.
Statement on ethics vote: There is a positive ethics vote.

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Lessons learned from EFSA AOP informed IATA case studies integrating *in vitro* data for DNT risk assessment

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Developmental neurotoxicity-DNT-refers to "any adverse effects on the normal development of the nervous system structure or function". Screening of chemicals for potential developmental neurotoxicity involves multiple and, in some cases, unknown targets and complex biological processes. The current regulatory testing paradigm is based on triggered DNT guideline *in-vivo* studies following prenatal and/or postnatal exposure. However, due to its complexity, cost and resource limitations, the need for development of NAMs (New Approach Methodologies) has been emphasized by regulatory agencies and stakeholders. Since 2014 EFSA has invested in NAMs for the DNT risk assessment of pesticides, aiming in a paradigm shift based on mechanistic understanding rather than testing for apical endpoints. EFSA elaborated an action plan and coordinated several activities, including a procurement for the implementation and interpretation of a DNT-*in vitro* testing battery (IVB) (Masjosthusmann et al., 2020), several workshop with stakeholders for the applicability in regulatory decisions and initiated a collaborative effort with OECD for the development of a guidance document for *in vitro* DNT testing.

Though the DNT-IVB has the potential to expand the DNT assessment in Integrated Approaches to Testing and Assessment (IATA) context, the regulatory confidence to make regulatory decisions is still at its infancy. To assess the applicability of the DNT-IVB for DNT hazard assessment in the context of the European pesticide regulations, EFSA developed two IATA case studies for two pesticides active substances (deltamethrin and flufenacet) based on hazard characterization problem formulation. In order to assure that the assessment provided a reliable information for the decision making, special considerations were given to the uncertainty analysis, with the identification, characterization and evaluation of uncertainties using a semi quantitative probabilistic approach. The IATAs were built in a structured evidence-based approach integrating all the available evidence including a systematic literature review, available guideline *in vivo* studies and data generated through the DNT-IVB. This stepwise approach culminated with the postulation of the evidence based AOP network for deltamethrin with a probabilistic quantitative estimation of the weight of evidence (WoE) using a Bayesian network analysis. The stressor-based approach was selected because the intention was to use the AOP conceptual framework to establish a causal relationship between the prototypical chemical deltamethrin exposure and the AO. For flufenacet, no DNT hazard was identified, hence, no AOP was postulated (Hernandez-Jerez et al., 2021).

The case studies are the first European experience integrating the DNT-IVB for regulatory purposes and showed the relevance of the DNT-IVB in an AOP informed IATA for regulatory decision-making. This work also allowed EFSA to draw some insights for its implementation: (1) Using an AOP informed IATA approach for integrating data of the DNT-IVB, the scientific validation is sufficient for the inclusion of NAMs; (2) The AOP informed IATA is fit for purpose for hazard identification and characterization problem formulation, e.g., equivocal *in-vivo* studies, additional information on MIEs or different test systems. (3) Lack of existing AOPs is a limitation and postulating and developing AOPs is resource demanding. A stressor based AOP is therefore facilitating facilitates this process, allowing for developing AOPs around specific pathways. Probabilistic quantification of the WoE supports the applicability of new AOPs development for which an OECD status is still not available (transparency, quantification of expert knowledge, causation…). Resource engagement remains an unresolved issue.

The collaborative effort, along with the demonstrated application using case studies, will build regulatory confidence in the application of NAMs for regulatory decision-making.

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Submission declaration:
Conflicts of interest: The corresponding author declares that there is no conflict of interest with the authors. The views expressed in this publication are those of the authors and should not be interpreted as representing the official position of the European Food Safety Authority (EFSA). EFSA assumes no responsibility or liability for any errors or inaccuracies that may appear.
Statement on ethics vote: There is a positive ethics vote.

Author Index

ALTEX Proceedings

https://proceedings.altex.org/

Vol. 10, No. 2 (2022)

Issued by
ALTEX Edition, Kreuzlingen, Switzerland

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