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Advances in Animal Models and Cutting-Edge Research in Alternatives: Proceedings of the Third International Conference on 3Rs Research and Progress, Vishakhapatnam, 2022

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Abstract

Animal experimentation has been integral to drug discovery and development and safety assessment for many years, since it provides insights into the mechanisms of drug efficacy and toxicity (e.g. pharmacology, pharmacokinetics and pharmacodynamics). However, due to species differences in physiology, metabolism and sensitivity to drugs, the animal models can often fail to replicate the effects of drugs and chemicals in human patients, workers and consumers. Researchers across the globe are increasingly applying the Three Rs principles by employing innovative methods in research and testing. The Three Rs concept focuses on: the *replacement* of animal models (e.g. with *in vitro* and *in silico* models or human studies), on the *reduction* of the number of animals required to achieve research objectives, and on the *refinement* of existing experimental practices (e.g. eliminating distress and enhancing animal wellbeing). For the last two years, Oncoseek Bio-Acasta Health, a 3-D cell culture-based cutting-edge translational

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biotechnology company, has organised an annual International Conference on 3Rs Research and Progress. This series of global conferences aims to bring together researchers with diverse expertise and interests, and provides a platform where they can share and discuss their research to promote practices according to the Three Rs principles. In November 2022, the 3rd international conference, *Advances in Animal Models and Cutting-Edge Research in Alternatives*, took place at the GITAM University in Vishakhapatnam (AP, India) in a hybrid format (i.e. online and in-person). These conference proceedings provide details of the presentations, which were categorised under five different topic sessions. It also describes a special interactive session on *in silico* strategies for preclinical research in oncology, which was held at the end of the first day.

Keywords
3Rs, animal-free, animal models, drug discovery, microphysiological system, NAMs, new approach methodologies, organ-on-a-chip, organs-on-chips, organoid, replacement, spheroid, Three Rs, tissue microenvironment

Introduction

The concept of the Three Rs (*replacement*, *reduction* and *refinement*) in animal experimentation was first introduced in 1959[1] and, since then, many technologies have been developed and introduced in order to replace animal models. In the last few decades, considerable advances have been made in human-derived and human-relevant models that mimic human physiology and drug disposition mechanisms[2,3] — for example, 3-D disease models that can improve the translation of preclinical data from *in vitro* studies to *in vivo* clinical trials. As summarised by Vangala et al.,[3] since the 1980s, there have been significant improvements in the application of the Three Rs principles, in the clinical relevance of the alternative models used and in ethical compliance. In addition, there is a global movement toward the adoption of the Three Rs principles in national and international policy and regulations for different stages of drug discovery.[4]

In the USA, the *Humane Research and Testing Act of 2021*[5] was introduced, which led to the establishment of the National Center for Alternatives to Animal Research and Testing, in order to promote the Three Rs principles in animal research and testing. Similarly, the *FDA Modernization Act* of 2021, which allows and encourages the use of "alternative testing methods to animal testing" was also introduced, in order to refine and reduce the use of animals for testing purposes.[6] Finally, on 29 September 2022, the US Senate unanimously passed the updated *FDA Modernization Act 2.0*, co-sponsored by Senators Rand Paul and Cory Booker. This bill permits drug developers to use non-animal methods alongside animal models to test toxicity when feasible. The bill authorises the use of certain alternatives to animal testing, including cell-based assays and computer models, to obtain an exemption from the US Food and Drug Administration (FDA) and thus allow the investigation of a drug's safety and efficacy through the use of non-animal methods. The bill also removes the requirement to use animal studies as part of the process to obtain a licence for a biological product that is biosimilar or interchangeable with another biological product. However, this US legislation does not put an end to animal testing completely.[7] Similarly, South Korea's new revised *Cosmetic Act* puts strict limits on the sale of cosmetics tested on animals, with the aim of helping to promote the use of alternatives to animal models in such testing.

The scientific, ethical and regulatory aspects of animal testing have taken great strides in adopting the principles of *refinement* and *reduction*. Moving forward, modern technologies, such as *'disease in a dish'* and *'organ-on-chips'* models, will be crucial to the goal of fully replacing animal models. Furthermore, in line with the Three Rs *replacement* principle, an extensive survey to determine the current use of animal-derived ingredients was undertaken by Cassotta et al.[8] From the survey findings, they recommended that the wider acceptance and use of alternative, non-animal ingredients should be actively promoted, in order to achieve full, rather than partial, *replacement*.

Oncoseek Bio-Acasta Health — an innovative Indian biotechnology start-up that is actively working to contribute to the progress of the Three Rs in research by using spheroid cell cultures to create *'disease in a dish'* models — has been organising a series of annual international conferences on '3Rs Research and Progress'. This conference is a great platform to bring together non-animal scientists/alternatives advocates, along with animal researchers, to exchange ideas and support the larger cause of replacing animal models with more effective, humane and translatable non-animal cell, tissue and computational experimental models. This article summarises the proceedings of the conference, which was held in hybrid format (i.e. online and in-person) on 17–18 November 2022. The inaugural event was held on

17 November, in the Shivaji Auditorium at GITAM University, Vishakhapatnam (India). The first day was an in-person event, where speakers delivered their lectures live and in-person; the second day was an online event, where speakers from overseas delivered their lectures virtually. The whole event was live streamed on a web platform, and a total of 27 speakers participated in the conference.

Conference sessions

The two-day conference included a keynote presentation on both days, and 25 talks which were categorised into the following six topic sessions, including a special interactive session at the end of the first day:

- Adoption of the 3Rs in research;
- Challenges associated with the use of alternative models;
- Alternative models for drug treatment;
- 'Disease in a dish' models;
- Carcinogenesis through spheroids; and
- *In silico* strategies for preclinical research in oncology (special interactive session).

All of the presentations are summarised under their respective topic sessions below, and are listed in Table 1.

Keynote lectures

Importance and application of the 3Rs in drug discovery and development

Dr Pratima Srivastava (Vice President, Discovery Biology Solutions, Aragen Life Sciences, India), delivered the first keynote lecture, emphasising the importance and application of the Three Rs in the drug discovery and development journey. Animal experiments are currently an integral part of the drug discovery and development process. They are used in various studies at the preliminary stages of the process, before the clinical stage is reached. However, the use of inappropriate animal models is one of the biggest causes of drug failure as they move toward Phase II. Non-animal models, tools and approaches can provide more physiologically relevant data as, often, the animal models do not reproduce the human situation completely, and thus this affects the translation of preclinical data to the clinical stage. A human-focused approach will help to improve accuracy and efficiency in the development of novel drugs and therapies. Research indicates that new approach methodologies (NAMs), which can include *in vitro* and *in silico* models, are the best way forward.

Much progress has been made in the field of alternative models — for example, 3-D systems such as spheroids and organoids, and organs-on-chips. In addition, the number and types of *in vitro* tests available have increased significantly. Another way of adopting the Three Rs principles is by modifying existing tests and studies. For example, *reduction* can be implemented by optimising the design of pharmacokinetic (PK) studies, and *refinement* strategies can improve the harm–benefit ratio for the animals. When searching for strategies to increase the number of new drugs coming through the drug development pipeline, implementation of the Three Rs principles should be given priority; the use of non-animal methods can lead to a decrease in turn-around time, the generation of better-quality data and increased cost-effectiveness. At different stages of the preclinical drug life cycle, wherever animal use was routine in the past, it is slowly being replaced with alternatives. Improved techniques and better research models can more accurately replicate human biological processes, and will facilitate the development of novel drugs and therapies with improved accuracy and efficiency. Evidence indicates that non-animal methods are the best way forward. The day when animal models are a thing of the past is hopefully not far away. These models will be replaced by research tools that mimic human physiology and biological processes (for example, organ-on-chip models).[9,10]

One Health threats and new approach (non-animal) tools for sustainable food systems

Dr Sandra Coecke (European Commission, Joint Research Centre (JRC), Italy) presented the second keynote lecture on one of the United Nations Sustainable Development goals — namely, the eradication of hunger — and on cutting-edge next-generation methods for sustainable food systems. She introduced a hierarchy of strategies for the eradication of hunger, including reducing food losses. Dr Coecke elaborated that food science, next-generation life science methods and artificial intelligence (AI) are all making significant impacts within the food industry. Many distinct areas of the market — from tackling food safety, security and sustainability issues, to promoting One Health and sustainable process practices in food production facilities, innovating new products and personalising product offerings through efficient and transparent regulatory processes — are benefiting from developments in food science and AI.

Food safety has always been a strong focus of the food industry. Failures in this area can have drastic impacts, given the implications for consumer health and safety due to the

Table 1. A Summary of the Conference Proceedings.

Presentation title	Presented by
Keynote lectures	
Importance and application of the 3Rs in drug discovery and development	Pratima Srivastava
One Health threats and new approach (non-animal) tools for sustainable food systems	Sandra Coecke
Session 1: Adoption of the 3Rs in research	
How to improve scientific quality and animal welfare when planning animal studies	Adrian Smith
Why must we replace animal experimentation and how do we do it?	Aysha Akhtar
Why do we fail to replace animal models with scientifically rational non-animal models?	Clive Roper
Choice of laboratory rodent diet may confound data interpretation and reproducibility	Sridhar Radhakrishnan
The 3Rs and ongoing efforts to meet deadlines	Balaji Bhyravbhatla
Coming together to accelerate the adoption of 3-D biology	Madhujit Damle
Evolved model systems	Venkat Koushik Pulla
Session 2: Challenges associated with the use of alternative models	
How to overcome systematic errors linked to R&D based on biomaterials and 3-D cell culture	Johannes Hackethal
Industrial adoption of integrated micro physiological systems: progress and challenges	Reyk Horland
Session 3: Alternative models for drug treatment	
Novel human hepatic technologies for the evaluation of human drug properties: Drug metabolism, drug–drug interactions, hepatotoxicity, and pharmacology	Albert P. Li
3-D bioprinted liver sinusoidal model: Focusing on the metabolic zonation	Falguni Pati
Tumor architecture governs therapeutic response: Case for 3-D spheroids for screening therapeutics	Manu Smriti Singh
Beating organs-on-chips as advanced preclinical tools for drug screening and disease modelling	Paola Occhetta
Novel human-relevant preclinical safety testing strategy for recombinant human monoclonal antibodies directed against foreign targets	Rohit Bisht
Alternative-to-animal models for pre-clinical evaluation of retinal therapeutics	Prajakta Dandekar
Improving translational relevance with humanized NSG mice	Krishna Bhagavatula
Session 4: 'Disease in a dish' models	
Studies on SARS-CoV-2, picornavirus, and human cytomegalovirus infections using human organoids	Dasja Pajkrt
Choosing the right liver *in vitro* model	Michael Johnson
Animals in the (Petri) dish: Towards a truly animal-free laboratory	Tilo Weber
Xeno-free bio-functional VitroGel system for 3-D cell culture and functional assays	John Huang
Organoids as a tool to explore the spectrum of human response to pathogens	Lisiena Hysenaj
Session 5: Carcinogenesis through spheroids	
The matrix is everywhere. It is all around us: Vindicating Morpheus through studies of cancer	Ramray Bhat
Special interactive session: *In Silico* Strategies for Preclinical Research in Oncology	
Computational approaches for translational oncology	Santosh Dixit
Dry lab challenges for wet lab scientists in preclinical oncology	Mandar Kulkarni
Data science applications to tissue microenvironment (TME) models	Shreekanth Joshi

presence of pathogens or toxic chemicals. In all food industry subsectors, it is important to increase the use of currently available *in vitro* and *in silico* cutting-edge alternative methods and to propose new approaches that can identify adverse health effects in existing and newly introduced foods worldwide. It was emphasised that next-generation (non-animal) food safety testing models and methods should be fully embraced, for the benefit of One Health. Different units of the JRC work collaboratively on food safety, security and sustainability, and on animal feed safety. The key drivers impacting food safety are: climate change; changes in food and farming systems; rapid technological advancements and emerging technologies; assessment of new technologies; the current COVID-19 pandemic; and the integration and improvement of hazard and risk assessment methodologies.

Dr Coecke has coordinated, on behalf of the JRC, an Organisation for Economic Co-operation and Development (OECD) guidance document on good *in vitro* practices.[11] She also managed the collective JRC input into a scoping document[12] that aims to develop scientific opinion on what tools could be used to overcome the barriers preventing the adoption of sustainable and healthy diets by consumers, to foster the necessary change toward

sustainability in the food environment. She further explained how NAMs can be used in a future framework for the application of *in vitro* metabolism models and quantitative *in vitro* to *in vivo* extrapolation (QIVIVE). In addition to being used for food safety and chemical risk assessment, NAMs can also be used to assess the effects of pathogens like SARS-CoV-2.[13–18]

Session 1: Adoption of the 3Rs in research

This session was conducted with a focus on the Three Rs principles in research, which recommends reducing animal use, refining animal experimentation practices focusing on animal safety and welfare, and eventually replacing animals in research. In this session, the speakers shared different guidelines that are available to improve scientific quality and animal welfare when planning animal studies, and underlined the urgency of adopting human-relevant and human-derived *in vitro* models. This session also highlighted various barriers to the adoption of the Three Rs in research.

How to improve scientific quality and animal welfare when planning animal studies

Prof. Adrian Smith (Norecopa, Norway) presented the PREPARE guidelines and discussed how best practice can be used to improve scientific quality and animal welfare when planning animal studies. He introduced Norecopa, which was established in 2007 (https://norecopa.no) and is Norway's National Consensus Platform for the Three Rs. Animal research is controversial, since it often involves taking healthy animals and subjecting them to treatment that causes pain, suffering, distress, or lasting harm. He talked about the strong legal and ethical incentives to implement the Three Rs principles in animal research. In addition, there are also very good scientific reasons for implementing them: animals that are in harmony with their surroundings will yield more valid data and it will be easier to detect the effects of treatment, without background noise caused by stress. Optimisation of animal research will also help to address the problems of poor reproducibility and translatability, which concern many scientists. We can do this by aiming for the best possible animal welfare, health and safety (for both animals and humans), a culture of care in research groups, and communication of best practices to others.

The pathway to improvement consists of many steps, which begin with adequate planning and end with a detailed report of the study and its findings. Frequently highlighted causes of the 'reproducibility crisis' are publication bias (reporting only positive results), lack of randomisation and blinding, low statistical power, *p*-value hacking (manipulating data to obtain significance) and 'HARK-ing' (Hypothesising After the Results are Known). The scientist may be more interested in the samples and their analysis, and may be less focused on conditions causing stress before and during the sampling process. Animals can also experience contingent suffering, for example, the stress from breeding, forming new social groups, transportation, acclimation to the research facility, allocation to an experimental group, adaptation to a new diet, handling and immobilisation. These are in addition to the pain and distress that may be caused by injections, gavaging and surgery. Better handling methods include the use of tunnels to pick up animals, or simply cupping them in the operator's hands, to reduce potential anxiety. Oral gavaging is another stress-inducing procedure that needs to be avoided. Nowadays there are methods whereby one can train mice to voluntarily take liquids. Clicker training can be used to avoid having to immobilise the animals.

The PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (https://norecopa.no/PREPARE) for better animal research were published in 2018[19] and these encourage scientists to collaborate with animal caretakers and technicians from Day 1 of planning. Prof. Smith elaborated on the importance and advantages of having a checklist, and gave examples from the aviation industry and from human surgeries (a surgical safety checklist). In addition, a contingency plan, based on risk assessment, is essential. Many of these contingency plans had to be revised during the COVID pandemic and lockdown (https://norecopa.no/be-prepared).

Like the Three Rs, the Three Ss (*Good Science, Good Sense* and *Good Sensibilities)* are also important. Good sensibilities refer to using one's heart, as well as one's head, when planning an experiment. This involves critical anthropomorphism: if a procedure would be unpleasant to a human being, we should assume that other vertebrates feel the same. Prof. Smith also talked about the culture of care and a closely related concept, the 'culture of challenge' (https://norecopa.no/coc). At the end of his talk, he touched upon the ARRIVE guidelines 2.0 for reporting animal experiments,[20] and concluded that if we 'PREPARE' well, we will 'ARRIVE' in better shape (Figure 1).[19,20]

Why we must replace animal experimentation and how do we do it?

Dr Aysha Akhtar (CEO, Center for Contemporary Sciences, USA),[21] presented a talk on catalysing human-specific medical research. She established the context of animal experimentation and its current status. Animal experimentation causes harm to more than 200 million animals globally each year,[22] and the USA is one of the top countries that perform animal experiments, with more than

Figure 1. Phases of animal research.
Reproduced with permission from Utrecht University (https://norecopa.no/PREPARE).

100 million animals used per year. Yet, most animals used in the USA are afforded no federal protection.[23,24] Despite the immense suffering caused by animal experimentation, it is defended by arguments that it is reliable, that animals provide sufficiently good models of human biology and diseases, and that, consequently, its use provides major human health benefits. However, a growing body of scientific literature raises important concerns about its reliability, as well as its predictive value for human outcomes and for understanding human physiology. A study published in the *British Medical Journal* in 2007 analysed the published animal and human data from six different treatments across five disease categories. It was found that only 50% of studies showed the faithful translation of animal data to clinical trials.[25] Dr Akhtar gave further examples of experiments on strokes and on HIV vaccines, which had more than 100 and 90 successful drug and vaccine candidates in animal experiments, respectively, but not one in human trials.[26]

The good news is that momentum is building, that will lead to a revolution in biomedical research, resulting in the replacement of animal testing, to the benefit of humans. Dr Akhtar further discussed various human-based research and testing methods currently in practice, including 3-D cultures, organ-on-a-chip/human-on-a-chip, organoids, imaging studies, biomarker studies, virtual humans and *in silico*/AI. She talked about the future of biomedical research and mentioned various legislative reforms over the last few years that support the use of more human-relevant methods of testing. She elaborated on the barriers to replacing animal testing — for example, miseducation, archaic policies, cultural bias and lack of funding for emerging technologies. The possible solutions to the above-mentioned barriers are to educate the next generation of scientists, update policies, change the narrative to demonstrate the important benefits that come from replacing animal testing, and increase investment and funding into emerging technologies. Dr Akhtar's organisation works to communicate recent data, which show that human-relevant science and testing methods are outperforming animal testing methods and can more faithfully translate to human clinical trials.[27]

Why do we fail to replace animal models with scientifically rational non-animal models?

Dr Clive Roper (Founder and Director, Roper Toxicology Consulting Ltd, UK), presented a talk about his consultancy company and discussed the reasons for failing to replace animal models with scientifically rational non-animal models. He talked about his experience in developing tests that are accepted for regulatory purposes and thus permit the replacement of animals, including tests for dermal absorption, ocular irritation and respiratory toxicology. Many companies that have developed non-animal test methods or models struggle to find customers. He explained that such methods and models will not get used unless they can help a pharmaceutical company to demonstrate the efficacy or safety of a new

drug, an agrochemical company to demonstrate the safety of a new pesticide, or a cosmetic company to show the safety of a new ingredient. From a regulatory perspective, the non-animal method, or model, developed should be able to provide information to screen out toxicity, protect animals, answer mechanistic questions, generate a safety assessment and demonstrate efficacy. Many regulatory animal tests result in a classification (e.g. Class 1, Class 2, or Not Classified). If a non-animal test is more predictive at measuring an endpoint than the current animal test, but cannot accurately predict this classification, it is not going to replace it. This is a regulatory and not a scientific question.

We often fail in our attempts to replace an animal test with a non-animal test by not adequately understanding the *in vitro–in vivo* correlation, including the consideration of species differences (human–animal correlation) and differences in study design. Thus, there are many benefits to be had in creating a very good screening test that can replace more animals, more quickly, than there is in trying to publish an OECD test guideline that may not gain regulatory acceptance (and, therefore, no *replacement*). Animal tests are required by law, but gaining information from the animal helps with identifying potential toxicities, optimising dose range finding and cancelling tests. Dr Roper also discussed the various stakeholders in non-animal methods or models, including the companies marketing the products, companies doing the testing, non-governmental organisations and regulatory agencies. A better understanding of the *in vitro–in vivo* correlation, validation system, tests and the stakeholders involved, can lead to successful formal validation.

Two different conceptual models were further discussed: the traditional inhalation model; and the human-relevant exposure-based inhalation model using human equivalent concentrations. He also summarised: an OECD case study based on the use of human computational fluid dynamics; aerosol simulation and the Epithelix MucilAir™ model (endpoint parameters, definitive study and the study results); and benchmark dose level modelling, the results of which are consistent with the biological understanding described in the relevant Adverse Outcome Pathway and which led to regulatory acceptance by the US Environmental Protection Agency and a waiver for the standard rodent test. Dr Roper concluded with a guideline containing 12 questions to consider when creating a new non-animal method, model, or test, and emphasised the importance of demonstrating the power of the model by using it for screening purposes, in order to obtain actual data to demonstrate its strengths and weaknesses.[28]

Choice of laboratory rodent diet may confound data interpretation and reproducibility

Dr Sridhar Radhakrishnan (Senior Scientist, Research Diets Inc., USA), presented his talk on how the laboratory rodent diet choice can alter data interpretation, potentially affecting reproducibility and knowledge gained within multiple fields of study. He started with the ARRIVE and PREPARE guidelines and their relevance. The reproducibility of experimental data is challenged by many factors in both clinical and preclinical research. In preclinical studies, several factors may be responsible, and diet is one variable that is commonly overlooked, especially by those not trained in nutrition. There are two main types of laboratory animal diets — grain/cereal-based diets (GBD; commonly referred to as 'chow diets') and purified ingredient diets. GBDs contain complex ingredients, each of which can provide multiple nutrients, as well as non-nutrients and contaminants; these ingredients may vary from batch to batch. Thus, even when choosing the same GBD as that used in the past by others, its composition will likely differ.

One class of non-nutrients is phytoestrogens — plant-based compounds that have pro-oestrogenic or anti-oestrogenic activity, as well as antioxidant and anti-cancer activity, etc. The most well-known phytoestrogens come from the isoflavone class, and are typically present in plants like soy and alfalfa. These plants are common ingredients in GBDs. Other non-nutrients include heavy metals, endotoxins, mycotoxins and synthetic toxicants (like pesticide residues), all of which can influence the phenotype of the animals. In contrast, purified diets are open-source formulas that contain refined ingredients. This offers the opportunity to closely control the composition, maintaining consistency from one batch to the next while minimising the presence of non-nutrients and contaminants. Unlike GBDs, purified diets are also suitable for imaging, since they lack chlorophyll that contributes to background fluorescent signalling. Dr Radhakrishnan suggested that, while GBDs and purified diets can be used in the same study, the resulting data should not be compared, as the diets are quite different in their composition and will likely have different effects on the phenotype. He presented the results of a study comparing the effects of two low-fat control diets, a GBD and a purified diet, relative to a high-fat purified diet. The results showed that, while body weight and epididymal fat remained similar on the two lower-fat diets, the changes in gut morphology were independent of dietary fat and were directly attributable to the fibre differences between the low-fat purified diet (~5% fibre; insoluble) and the GBD (~15–20% fibre; mixture of soluble and insoluble). The refined and open-source nature of purified diet ingredients also allows modification of the different nutrients (fat level, sucrose, cholesterol, etc.), in order to formulate diets for the study of metabolic diseases and other diet-induced phenotypes, including obesity, NAFLD, hypertension, metabolic syndrome and atherosclerosis.[29–31]

The 3Rs and ongoing efforts to meet deadlines

Dr Balaji Bhyravbhatla (Managing Director, Hylasco Bio-Technology Pvt Ltd, India), presented a talk on the

importance of identifying the correct types of animals to be used in preclinical experiments and on the best practices involved. Ways to reduce animal use in preclinical testing is an ongoing debate, and stakeholders generally agree that other species (i.e. rodents and non-rodents) should not be used to advance the wellbeing of humans. This moral dilemma is not lost on the researchers and other animal users. However, it is important to note that preclinical animal use has contributed immensely to the understanding of medicine and biology overall, and therefore has a very direct impact on the health and wellbeing of mankind. A rational approach to the Three Rs, while choosing conventional or specific pathogen-free (genetically and health-wise standardised) animals for preclinical studies, is considered essential to the validity of the data obtained. Dr Bhyravbhatla elaborated on the advantages of obtaining animals from commercial breeders and the criteria for breeding. Commercial breeders, such as Hylasco, give biosecurity utmost importance. Thus, they place increasing importance on laboratory animal quality control (QC) in breeding, for example, genetically engineered mice (the immunodeficient nude, SCID and triple immunodeficient mouse models), and health monitoring QC to reduce the number of animals used.

Coming together to accelerate the adoption of 3-D biology

Dr Madhujit Damle (General Manager, India and South Asia, Molecular Devices, USA), presented a talk on various 3-D model systems currently in use, and introduced a model for 3-D neurite outgrowth assessment and automated organoid screening workflows. Molecular Devices develops a broad portfolio of biological research tools to understand life at a cellular and molecular level. With their experience in drug discovery and development, GxP compliance and workflow automation, in partnership with their customers they accelerate the development of novel therapeutics. Dr Damle discussed the potential of 3-D models to improve the drug discovery paradigm and the barriers to switching from 2-D to 3-D model systems. The poor physiological relevance of *in vitro* models of the nervous system is an important limitation in understanding mechanisms of neurological diseases and consequently drug development. Molecular Devices has developed a model for 3-D neurite outgrowth assessment by using iPSC-derived neurons in the microfluidic, high-throughput OrganoPlate® platform. It is a relatively simple 3-D model system with manual workflow, with imaging and 3-D analysis enabling the quantification of the findings. Further, he summarised various organoid culture approaches, including ECM (matrigel/hydrogel), scaffold-free (ULA U-bottom plates) and organ-on-chips, and introduced the customisable automated organoid screening workflow developed by Molecular Devices. He discussed the unique position of Molecular Devices to address key challenges, along with their collaborators, and emphasised the importance of collaboration between the model system provider and the customer in driving forward the standardisation of protocol elements and finding the optimal balance between standardisation and flexibility.

Evolved model systems

Dr Venkat Koushik Pulla (Commercial Marketing Manager, Merck Life Sciences, India), presented various evolved model systems, their relevance and their advantages. 3-D cell culture is a more biologically relevant model system, as it better recapitulates the *in vivo* environment and physiology compared to 2-D. He described various 3-D cell culture tools, focusing on the specifications and on the advantages of synthetic and natural hydrogels. He introduced the concept of ready-to-use TrueGel3D HTS Hydrogel Plates and 3-D organoids, which saves considerable time and resources. Another dimension of mimicking natural physiological conditions involves the development of dynamic systems with constant influx of nutrients (and reagents) and removal of waste products. In connection with this attribute, Dr Pulla introduced CellASIC Onix microfluidic system, which enables long-term imaging with fewer reagents, while modulating critical parameters (like oxygen, temperature, osmolarity, humidity and pH).

Session 2: Challenges associated with the use of alternative models

The replacement of animal testing faces various barriers, including miseducation, archaic policies, cultural bias and lack of funding and investment. It is also impeded by scientific barriers and systematic errors linked to R&D, as described below. This session consisted of talks that discussed some of the challenges associated with the use of non-animal alternative models and provided recommendations to overcome the said challenges.

How to overcome systematic errors linked to R&D based on biomaterials and 3-D cell culture

Dr Johannes Hackethal (Founder and CEO, THT Biomaterials GmbH, Austria) presented his talk on the different platform technologies developed by his company to overcome systematic errors linked to R&D. There is a critical unmet need for representative *in vitro* models for human cells for medical purposes, or functional human tissues to support or replace injured tissues or organs *in vivo*. While many synthetic and natural non-human materials are already established for use in 2-D and 3-D applications, they still do not mimic the complex functions of the sum of the

extracellular matrix (ECM) in native, intact human tissue. Dr Hackethal discussed the main challenges to the introduction of new platforms technologies, and the strategies and drivers used by his company. He elaborated on various applications of THT Biomaterials — for example, 2-D and 3-D cell culture, hydrogels, *in vitro* studies, lab-on-chips, bioprinting, tissue engineering, bioinks, organoids and stem cells. He described strategies to isolate ECM proteins from the human placenta, in order to develop a platform technology for applications in cell culture and regenerative medicine. In these methods, atelocollagen-I and other subtypes were isolated by pepsin digestion, followed by salt precipitation and further purification steps. Moreover, HUMAN PLACENTA Substrate® (hpS), which comprises a liquid mixture of various ECM proteins and native laminin-111, was obtained by Tris-NaCl buffer extraction and further processing. The products were characterised by various photometric or antibody-based methods.

Various 2-D and 3-D *in vitro* cell culture experiments were performed with different cell types. In 2-D *in vitro* experiments, cells cultured in human ECM protein-coated plates showed a higher viability rate compared to cells cultured in plates coated with bovine- or porcine-derived materials. With regard to 3-D cell culture: human umbilical vein endothelial cells (HUVECs), cultured in a mix of hpS and fibrinogen, formed randomly oriented 3-D cell networks after approximately one week in culture; colon organoids of cells isolated from malignant carcinomas were grown; and HepG2 cells were successfully bioprinted by using a mix of hpS with alginate. The company has 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 2-D and 3-D *in vitro* assays (e.g. 3-D bioprinting, cancer or toxicity studies), and possibly also for *in vivo* applications. However, more research is necessary to assess the full potential of this human placenta platform technology for TERM.

Industrial adoption of integrated micro physiological systems: Progress and challenges

Dr Reyk Horland (CEO of TissUse, Germany) presented his talk on the current status of the adoption of microphysiological systems (MPS) and their importance. Traditional drug testing still leads to dramatic failure rates in clinical studies. MPS have proven to be a powerful tool for recreating human tissue-like and organ-like functions at the research level, providing the basis for the establishment of qualified preclinical assays with improved predictive power. However, industrial adoption of MPS and respective assays is progressing slowly due to their complexity. In the first part of the presentation, he highlighted examples of the established single-organ chip, two-organ and four-organ chip solutions. He explained the underlying universal microfluidic multi-organ-chip (MOC) platform that is the size of a microscope slide, integrating an on-chip micro-pump and capable of interconnecting different organ equivalents. TissUse can customise the platform to different organ models and have a flexible fit-for-purpose assay set-up. Dr Horland also described the advantages of automation, including redundancy and repeatability, high-throughput testing, high-content data, physiological maintenance and reduction of working hours.[32] Further, he discussed issues associated with ensuring the long-term performance and industrial acceptance of MPS, such as design criteria, tissue supply and on-chip tissue homeostasis.

The second part of the presentation focused on case studies of MOC use in the pharmaceutical industry. The first example discussed was the TissUse bone marrow-on-a-chip setup.[33] The second example was the two-organ combination of liver and skin for the cosmetic industry (liver and skin for toxicokinetics and toxicodynamics). Studies were performed with this two-organ combination to compare systemic *versus* topical application, and single *versus* repeated doses of different test compounds, such as retinoic acid and permethrin, with high intra-laboratory and inter-laboratory reproducibility.[34,35] Dr Horland also highlighted models of the intestine, liver, kidney, brain and blood–brain barrier (BBB) for multi-purpose use, with a focus on absorption, distribution, metabolism and excretion (ADME) profiling, pharmacokinetics, organ-specific toxicity and BBB permeation of drug candidates.[36,37] Finally, he outlined a roadmap to bring these assays into regulatory-accepted drug testing on a global scale.[38,39]

Session 3: Alternative models for drug treatment

Given the historical role played by animals in the drug discovery and development journey, significant progress has recently been made with alternative models. These models can reduce the use of animals in research and help to refine animal experimentation, in order to obtain relatively better translational data. *In vitro* human-based experimental systems that can replicate human *in vivo* organ-specific functions are valuable preclinical tools for the assessment of human-specific drug properties. This session discussed various novel *in vitro* models for the evaluation of different human drug properties.

Novel human hepatic technologies for the evaluation of human drug properties: Drug metabolism, drug–drug interactions, hepatotoxicity and pharmacology

Dr Albert P. Li (CSO Pharmacology and Toxicology, Discovery Life Sciences LLC, USA) presented his lecture on novel hepatic technologies for the evaluation of human

drug properties. The clinical trial failure rate for drug candidates, that were found to be acceptable in preclinical trials on non-human animals, has been reported to be greater than 90% — an observation that is often attributed to species differences in drug metabolism, toxicity and efficacy. *In vitro* human-based experimental systems that can replicate human *in vivo* organ-specific functions are valuable preclinical tools for the assessment of human-specific drug properties. The key desirable properties of these *in vitro* experimental systems are their organ-specificity and species-specificity.

Dr Li highlighted the discovery focus of Discovery Life Sciences LLC, namely the liver and intestine. He further described the properties of their two major novel human *in vitro* technologies — 999 Elite Cryopreserved Human Hepatocytes and MetMax Cryopreserved Human Hepatocytes — and outlined their application in the evaluation of human drug properties during drug development. The 999 Elite Cryopreserved Human Hepatocytes are optimally cryopreserved, in order to retain > 90% viability and near 100% attachment, to form > 90% confluent cultures that are stable for at least nine days, with some preparations forming stable confluent cultures for over 40 days. These cell cultures can be used for the evaluation of transporter-mediated drug uptake and efflux, P450 inhibition and induction, and hepatotoxicity. Recent applications include an evaluation of the extent and duration of efficacy of gene therapy modalities, as well as in basic research on liver disease (such as fibrosis and non-alcoholic fatty liver disease), including the development of therapeutic approaches.

The MetMax Cryopreserved Human Hepatocytes are permeabilised cells which, when supplemented with metabolic cofactors, retain all key drug metabolising enzyme pathways at the same activities as intact cryopreserved human hepatocytes. MetMax Cryopreserved Human Hepatocytes represent a convenient experimental model that can be stored in a –80°C freezer and used directly after thawing, thereby eliminating requirements for liquid nitrogen storage and the laborious procedures for cell recovery that are required for conventional cryopreserved hepatocytes. MetMax Cryopreserved Human Hepatocytes are compatible with higher throughput instrumentation, and can be applied to the screening of new chemical entities for key drug properties, such as metabolic stability and P450 inhibitory potential. Additional advantages of using MetMax Human Hepatocytes include the quantification of drug metabolism at cytotoxic drug concentrations, and evaluation of the role of drug-metabolising enzyme pathways via cofactor specification as part of a drug's properties. A major novel application is the identification of a drug candidate's potential to cause drug-induced liver injury (DILI), based on cytotoxic reactive metabolite formation via the metabolism-dependent cytotoxicity assay. In such studies, MetMax Human Hepatocytes are used as an exogenous metabolic activating system, with cytotoxicity evaluated in HEK293 cells (a cell line devoid of P450 activities). Cytotoxic reactive metabolite formation is identified by an increase in cytotoxicity in the presence of NADPH, indicating metabolic activation, and attenuation of cytotoxicity by reduced glutathione, a known detoxifying cofactor of reactive metabolites. Thus, 999 Elite and MetMax Cryopreserved Human Hepatocytes can be routinely used for the evaluation of human-specific drug properties during drug development.[40–45]

3-D bioprinted liver sinusoidal model: Focusing on the metabolic zonation

Dr Falguni Pati (Associate Professor, Department of Biomedical Engineering, IIT Hyderabad, India) described the development and design of a 3-D printed mini bioreactor that mimics the arrangement of hepatocytes and the sinusoidal network *in vivo*. He touched on the fact that the liver is the largest organ in the human body, performing a wide range of metabolic functions, and that it is the primary organ involved in drug metabolism. Further, he described at length an interesting phenomenon observed in the liver — namely, metabolic zonation — in which there is a spatial separation of metabolic functions, and explained its relevance to the proper functioning of the hepatocytes under physiological conditions. Metabolic zonation is modulated by the gradient of oxygen, hormones, nutrients, metabolites and cytokines. The native microenvironment and the spatial arrangement of hepatocytes are important parameters to be considered when developing an *in vitro* model, as different aspects of liver function — such as drug metabolism and disease onset and progression — are zone dependent. This consideration is somewhat lacking in many of the current models. Dr Pati described the decellularised liver matrix hydrogel developed by his team to mimic the native microenvironment, and explained how they designed and 3-D printed a mini bioreactor that mimics the arrangement of hepatocytes and the sinusoidal network *in vivo*. A series of biological characterisations and functional assessments were conducted, which confirmed functional heterogeneity throughout the zones of the bioreactor. The perfusion of cell culture medium mimics the blood flow, and allows the exchange of nutrients and oxygen between the medium and the cells, leading to the establishment of different metabolic zones.

Tumour architecture governs therapeutic response: Case for 3-D spheroids for screening therapeutics

Dr Manu Smriti Singh (Associate Professor, Department of Biotechnology and Center of Excellence for Nanosensors and Nanomedicines, Bennett University, India) presented a talk on the 3-D spheroid-based mice tumour model

developed through her research. She started by describing the complexity of cancer and the relevance of the tumour microenvironment (TME), while considering the patient-relevant animal tumour model. Her team developed and optimised the implantation protocol for a 3-D spheroid-based mice tumour model, based on injecting a single preformed mini-tumour. They simultaneously compared this 3-D tumour model with the routinely used 2-D model, wherein single cells are injected *en masse*. Further, they compared the growth kinetics of 3-D and 2-D-based mice tumour models, in addition to analysing their clinical correlation and pathological characterisation. The two models differ characteristically with respect to blood vasculature ($CD31^+$) and carcinoma-associated fibroblasts (α smooth muscle $actin^+$) cells and the ensuing TME dynamics. Two formulations of the doxorubicin drug, i.e. Doxil (nanoparticle) and Avastin (humanised antibody), were tested in the two tumour models. This study highlighted the significance of tumour histopathology and the role of supporting non-cancer cells of the TME, in determining the outcome from different classes of therapy. It demonstrated the superiority of 3-D spheroids, and showed the value of screening anti-cancer therapies in a more physiologically relevant setting.[46]

Beating organs-on-chips as advanced preclinical tools for drug screening and disease modelling

Dr Paola Occhetta (CEO, BiomimX Srl, Italy) delivered a talk on new beating organs-on-chips (OoCs), which are advanced platforms integrating native-like 3-D mechanical microenvironments with an unprecedented level of precision. The poor translational value of current preclinical tests contributes to the failure rate in drug development, which is still over 98%. OoCs have recently emerged as innovative *in vitro* tools, holding the potential to improve the prediction of human drug responses by replicating human physiology and pathology with unprecedented precision. BiomimX leverages beating OoC technology to unlock preclinical models reflecting clinical complexity, aiming toward the design of treatments tailored to patients. This is achieved through uBeat®, an innovative reliable, tunable and versatile technology that allows the modulation of the mechanical deformation exerted on 3-D microtissues in a controlled fashion.[47] Two uBeat-based models were presented: i) uHeart, a beating heart-on-a-chip integrating real-time electrophysiological measurements;[48] and ii) uKnee, the first *in vitro* model of human osteoarthritic (OA) cartilage-on-a-chip.[49]

uHeart provides 3-D human cardiac microtissues with a physiological cyclic uniaxial strain (10%, 1 Hz),[50] and can be used to develop either physiological or pathological models. In the first case, cardiomyocytes from human induced pluripotent stem cells and human fibroblasts are embedded in a fibrin hydrogel and cultured within uHeart by using a heartbeat-like mechanical training to develop synchronously beating cardiac microtissues, as confirmed by on-chip electrophysiology studies.[51] Cardiac pathological models have also been developed by tuning mechanical environments, demonstrating the versatility of uHeart in modelling cardiac fibrosis (uScar)[52,53] and inherited cardiomyopathies, among others.

uKnee provides 3-D cartilage-on-a-chip with hyper-physiological (HP) compression (30%, 1 Hz), sufficient to elicit osteoarthritis (OA) pathogenesis *in vitro*.[54] Healthy cartilage-on-a-chip was first generated from human articular chondrocytes embedded in a poly(ethylene glycol)-based hydrogel. HP-stimulated cartilage-on-a-chip showed a shift in cartilage homeostasis toward catabolism, inflammation and hypertrophy, and the acquisition of a gene profile compatible with OA clinical evidence. They characterised several cartilage-related genes and genes related to the joint formation during development.[55] uKnee demonstrates that the recapitulation of all relevant hallmarks (i.e. altered mechanical environment in the case of OA) of a pathology is essential to obtain clinically relevant profiling of new therapies.

Both uHeart and uKnee models were successfully exploited for testing the effects of well-known drugs and innovative compounds.[56] The integration of beating OoCs, powered by uBeat, in a 3-D mechanical microenvironment resulted in OoC models with enhanced functionality and resemblance to pathological states. Beating OoCs are highly versatile and applicable to any organ in which mechanical stimulation exerts a pathophysiological state, representing new powerful tools for *in vitro* drug screening and disease modelling (Figure 2).

Novel human-relevant preclinical safety testing strategy for recombinant human monoclonal antibodies directed against foreign targets

Dr Rohit Bisht (Science Research Associate, Department of Science-Regulatory Toxicology, People for the Ethical Treatment of Animals (PETA), India) presented a novel human-relevant, animal-free preclinical safety testing strategy for recombinant human monoclonal antibodies (mAbs) directed against foreign targets. As shown in Table 2,[57] the safety profile for a drug in this class indicates low risk to humans, and the non-animal safety testing strategy was developed to fully mitigate the two 'low' risks of off-target binding and immunogenicity. The preclinical strategy is intended to support a single-dose, first-in-human (FIH) clinical trial for any recombinant human mAb with a foreign target, by prioritising *in vitro* and *in silico* techniques that robustly address relevant

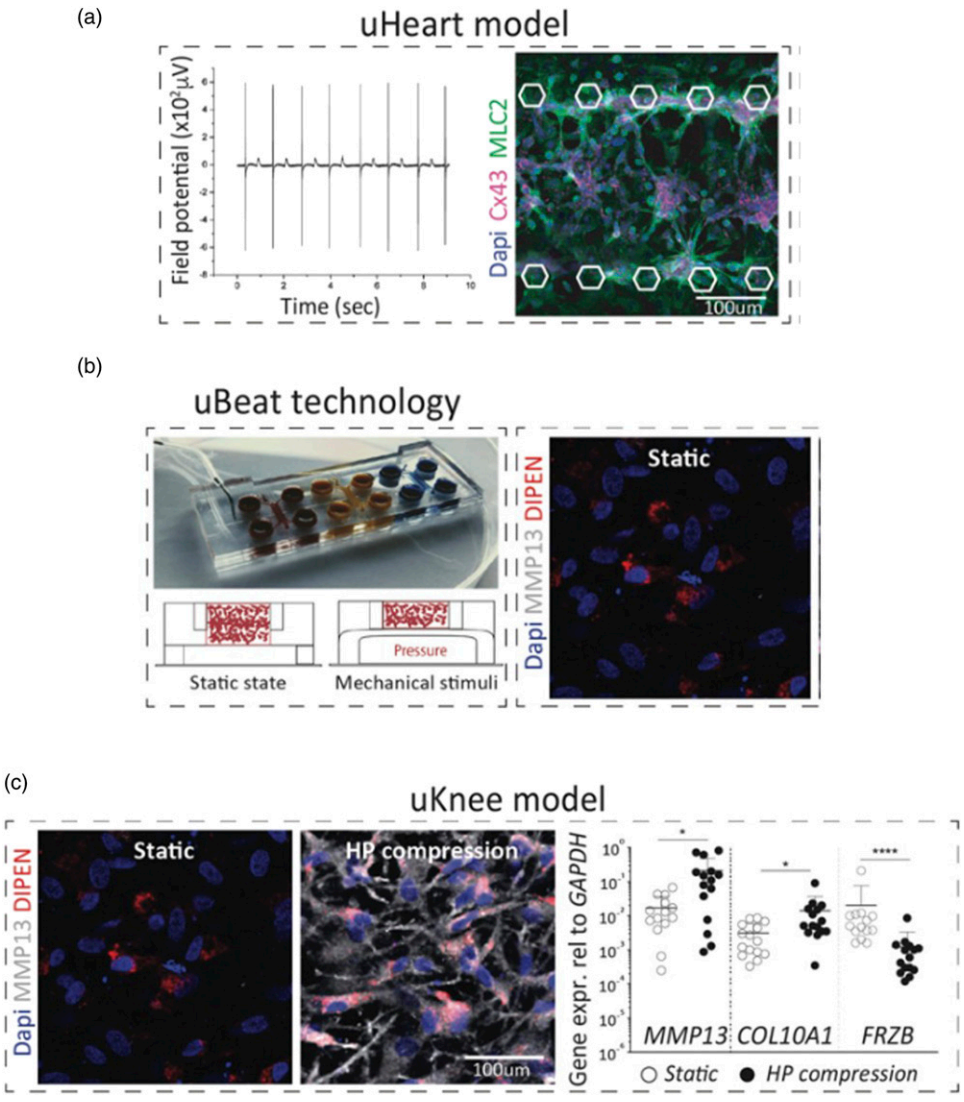

Figure 2. Novel organ-on-a-chip models.

Table 2. The potential human toxicity risks specific to the target drug class.

Potential risk	Degree of risk for target drug class	Rationale
Excessive on-target activity (exaggerated pharmacology)	Negligible	Drug within the target class has high specificity/affinity for an antigen foreign to humans.
Off-target binding	Low (to be fully mitigated by preclinical strategy)	Low risk for antibody with high specificity/affinity for non-human target.
Genotoxicity, carcinogenicity, reproductive toxicity	Negligible	Testing for these is not recommended by ICH S6 (R1)[57] for this drug class.
Toxic metabolite or reactive intermediate	Negligible	As per ICH S6(R1),[57] the metabolism of therapeutic proteins is well-understood and generates no safety risk.
Immunogenicity	Low (to be fully mitigated by preclinical strategy)	Unwanted human immune response is unlikely for a fully human antibody with a non-human target.

human safety concerns. The package of methods will act as an important assessment tool that is expected to safeguard human health and overcome limitations associated with animal models.

Immunogenicity will be investigated through a tiered strategy that starts with an *in silico* assessment. The *in silico* modelling platform will assess the mAb sequence and predict its binding of peptides to human MHC class II alleles.[58] If the *in silico* binding predictions indicate low risk, the mAb will be considered unlikely to cause a human immune response. In contrast, in case of *in silico*-predicted high risk, the mAb will be further screened for immunogenicity with human cell-based assays that measure a panel of cytokines through a multiplex cytokine ELISA. For off-target binding risk assessment, an immunohistochemical tissue cross-reactivity study, employing a panel of human tissues and a human proteome array technique, will be used in parallel. The methods used for the immunohistochemical tissue cross-reactivity technique are based on the recommendations in the 1997 US FDA/CBER *Points to consider in the manufacture and testing of monoclonal antibody products for human use* document, and in Annex II of *Directive 75/318/EEC* (i.e. *Production and quality control of monoclonal antibodies*).[59,60] The cell-based array data will provide orthogonal off-target binding data with information on mAb binding to thousands of human proteins expressed in a native human environment. All the assays for both risk categories must implement appropriate positive, negative and benchmark controls, to enable risk assessment.

A drug class-specific testing approach is in line with legislation and policy guiding regulatory agencies to prioritise modern testing approaches that safeguard human health while replacing animal-based experiments; International Council for Harmonisation (ICH) guidance recommends a significant reduction in animal-based testing when evaluating the preclinical safety of a therapeutic mAb directed against a foreign antigen.[61] Recently, Indian regulatory bodies have also taken significant steps toward the replacement of animal-based methods with alternatives in the interest of the Three Rs principles and regulatory harmonisation. The Ministry of Health and Family Welfare, Government of India, proposed an amendment to the *New drugs and clinical trials rules 2019*, that includes animal-free testing methods for the preclinical testing of new drugs.[62] The Indian Pharmacopoeia Commission accepted PETA India's recommendations, and established a new expert working group for alternatives to animal testing. The Indian Council for Medical Research (ICMR) stated, in a vision paper, that animal data are not being translated to successful clinical outcomes, as evident from failure rates of over 90% between nominations for Phase I clinical trials and new drug approvals. Hence, there is a need to make India self-reliant in the development of human-relevant, animal-free techniques.[63]

Despite these efforts toward drug development modernisation, it is unclear how regulatory agencies will respond to preclinical data from an exclusively non-animal approach for a mAb in the target drug class. PETA scientists and partners therefore developed a case study candidate therapeutic, to enable targeted discussion with regulators.[64] The candidate is a combination of two recombinant human mAbs that target diphtheria toxin, and it exemplifies the type of well-characterised, purified and highly specific product to which the non-animal strategy may be applied. In addition to providing an ideal case study for the non-animal preclinical strategy, the Diphtheria Antitoxin Recombinant Human Monoclonal Antibody (DATMAB) candidate offers an alternative to the existing treatment for diphtheria. The currently available treatment, diphtheria antitoxin (DAT), is based on polyclonal equine serum and has numerous disadvantages, including serum sickness, batch-to-batch variation in quality, severe supply scarcity and the use of animals for production.[65] Since each DATMAB will be fully human and sequence-defined, the therapeutic is expected to overcome these disadvantages. DATMABs are in the preclinical phase of development, and PETA scientists are engaged in regulatory discussions about the non-animal preclinical testing strategy as it applies to DATMABs. Dr Rohit is in communication with Indian regulators, government scientific agencies, government testing laboratories and stakeholder companies, and PETA scientists are also holding formal meetings with the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Initial regulatory feedback indicates that the strategy appears reasonable.

Alternative-to-animal models for pre-clinical evaluation of retinal therapeutics

Dr Prajakta Dandekar (Assistant Professor, Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, India) presented the diverse non-animal models that have been developed by her team for the preclinical evaluation of therapeutics under development for the treatment of retinal diseases. She described the current status of retinal diseases, which present a significant global public health concern, their available treatments, drug development, and the conventional methods used for this process. As of 2015, only 13.8% of drugs across all indications were estimated to have progressed from Phase I clinical trials to approval. This is proposed to be primarily due to the lack of suitable preclinical models that can fully recapitulate human pathophysiology. Dr Dandekar discussed the following alternative models developed by her team:

— An *in vitro* 3-D spheroid model, developed with choroid-retinal vascular endothelial cells, to enable the recapitulation of diabetic retinopathy (Figure 3). This model has been used to study angiogenesis *in vitro*, as well as the individual

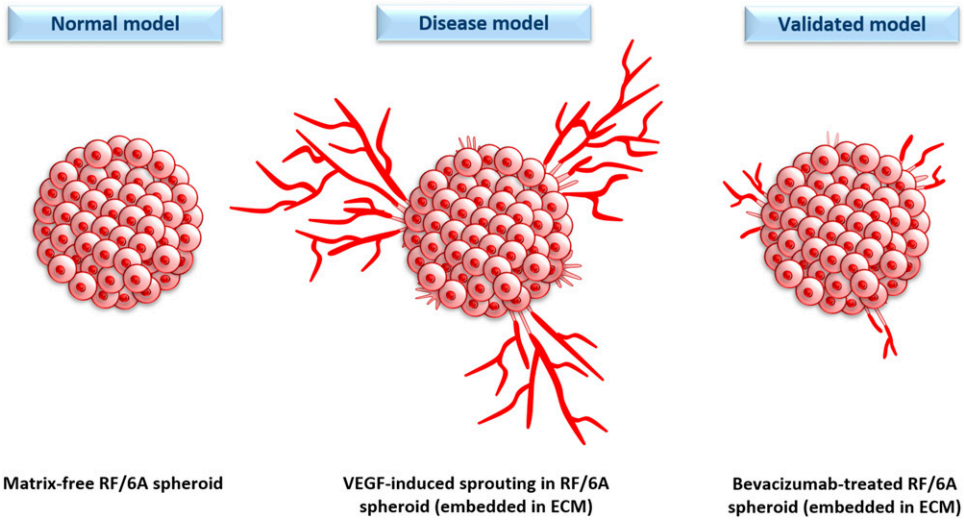

Figure 3. A 3-D spheroid model developed with choroid-retinal vascular endothelial cells to enable the recapitulation of diabetic retinopathy.
RF/6A cells = monkey choroid-retinal vascular endothelial cells; ECM = extracellular matrix.

and combined effects of VEGF and an anti-VEGF monoclonal antibody (bevacizumab) on cellular proliferation and 3-D endothelial sprout formation.

— A triple coculture of retinoblastoma, retinal epithelium and choroid endothelial cells, developed by using a protein coating cocktail, was the basis of an *in vitro* retinoblastoma model that closely resembles the microenvironment of the eye. This triple coculture model was used for screening drug toxicity, based on the growth profile of retinoblastoma cells, with carboplatin used as the model drug. A combination of bevacizumab and carboplatin was evaluated by using this model, to lower the necessary therapeutic concentration of carboplatin, and thereby reduce its physiological side effects.

A proof-of-concept, perfusion-based microfluidic device was also developed by the team. The device was initially used for supporting cocultures of retinal epithelium and precursor cells. The barrier integrity of this coculture system was confirmed by evaluating the permeability of fluorescently labelled molecules, wherein improved barrier function was observed, as compared to a static model. The coculture expressed characteristic phenotypic protein markers for both cell types. Studies confirming the functionality of this retinal coculture model have highlighted the potential use of microfluidic-based coculture models for replicating retinal diseases (Figure 4), as well as for culturing spheroids under perfusion conditions.[66,67]

Improving translational relevance with humanised NSG mice

Dr Krishna Bhagavatula (Director, Business Development, The Jackson Laboratory, USA) described the process of generating and characterising humanised mice, and the preclinical research applications of these novel platforms. Dr Bhagavatula started his talk with the mission of The Jackson Laboratory — to discover precise genomic solutions for diseases, and empower the global biomedical community in the shared quest to improve human health with a focus on discovery, innovation, research and education.

A range of NSG™ mouse model variants are available from The Jackson Laboratory. The NOD (Non-Obese Diabetic) background in the NOD *scid* gamma (NSG) genotype contributes to the absence of haemolytic complement, reduced dendritic cell function, defective macrophages and optimal human haematopoietic stem cell engraftment. The *scid* mutation in NSG prevents the development of mature T-cells and B-cells, and the IL-2 receptor common gamma chain (IL2Rg) deficiency eliminates signalling from six distinct interleukins (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) and blocks NK-cell development. Together these features accelerate the ability of NSG mice to engraft human cells. Some factors to consider while choosing NSG mice as hosts are radiation sensitivity and possible higher toxicity of some genotoxic drugs.

Humanised NSG (Hu-NSG) mice are also available, including those humanised with CD34 (enriched with CD34 haematopoietic stem cells) or those humanised with peripheral blood mononuclear cells. Dr Bhagavatula briefly explained the process of generating Hu-NSG mice, and highlighted that females engraft more efficiently than males. Further, he briefly highlighted other humanised mouse models available on the NSG platform.

There are several basic biological and preclinical research applications for NSG mice, including: human

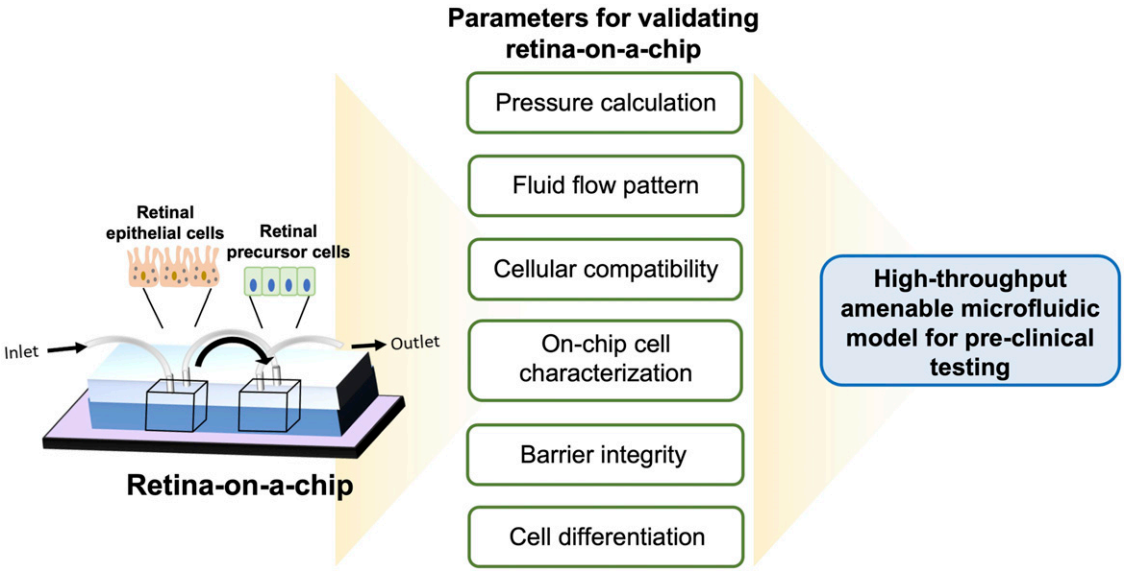

Figure 4. Proof-of-concept of the retina-on-a-chip model, for use as a preclinical tool.

haematopoiesis, stem cells/regenerative medicine, graft *versus* host disease, infectious disease and immuno-oncology. An example of an application of NSG mice in regenerative medicine is the creation of blood progenitors from human fibroblasts by using NSG mice. The use of NSG mice (hu-CD34 engrafted) to create a humanised dengue model, through infection with a DENV-2 strain via mosquito bite or intradermal injection, is an example of the application of NSG mice in infectious disease research.

Onco-Hu NSG mice are used for immune modulation studies. Studies conducted on three different cancers in The Jackson Laboratory show that the humanisation of NSG mice has no significant impact on patient-derived xenograft (PDX) growth kinetics. PDX growth is not grossly affected by HLA type matching. Tumour growth kinetics are similar in humanised and non-humanised hosts. Approximately, 15% of PDX tumours fail to grow in humanised mice. Human immune effector cells (lymphocytes) infiltrate human tumours in Hu-CD34-NSG and Hu-CD34-SGM3 mice (Onco-Hu mice). The Onco-Hu mice PDX has been shown to respond to anti-tumour agents (e.g. anti-PD-1, anti-CTLA-4, anti-OX40 and anti-GITR).[68–82]

Session 4: 'Disease in a dish' models

There is no single *in vitro* method that fully meets the needs of every research question, as *in vitro* models are a trade-off between *in vivo* relevancy, cost and throughput. To better mimic human physiology for a particular disease condition, a higher level of complexity was sought for a number of alternative models, leading to the concept of 'disease in a dish'. This session discusses various aspects of 'disease in a dish' models, including the use of organoids/spheroids to further our understanding of certain infections and the pathogenic effects. It also emphasises the importance of choosing the right model for the study of a given disease.

Studies on SARS-CoV-2, picornavirus and human cytomegalovirus infections using human organoids

Professor Dasja Pajkrt (Viral Pediatric Infectious Diseases, Amsterdam University Medical Centers, University of Amsterdam, The Netherlands) described studies conducted on SARS-CoV-2, picornavirus and human cytomegalovirus infections by using human organoids. She talked about the structure, species classification and pathogenesis of picornaviruses. The following questions regarding pathogenesis were covered: initiation of virus infection, virus migration to the secondary site(s), what happens at the secondary site(s), and the determinants of susceptibility. Virus research has historically relied on research with cell lines or animal models. Organoid technology is highly applicable in the virology field, yet it remains unexplored. Organoid systems can mimic the *in vivo* human physiological environment and provide tools to study human host–virus interactions. The pathogenesis of a variety of human viruses, such as SARS-CoV-2, picornaviruses and human cytomegalovirus (CMV), is increasingly being studied by using these novel human organoid models.

Professor Pajkrt's laboratory has developed various organoid systems, namely: human airway epithelium (HAE), human intestinal epithelium (HIE) and whole brain organoids. HAE cultures and lung organoids permit host–

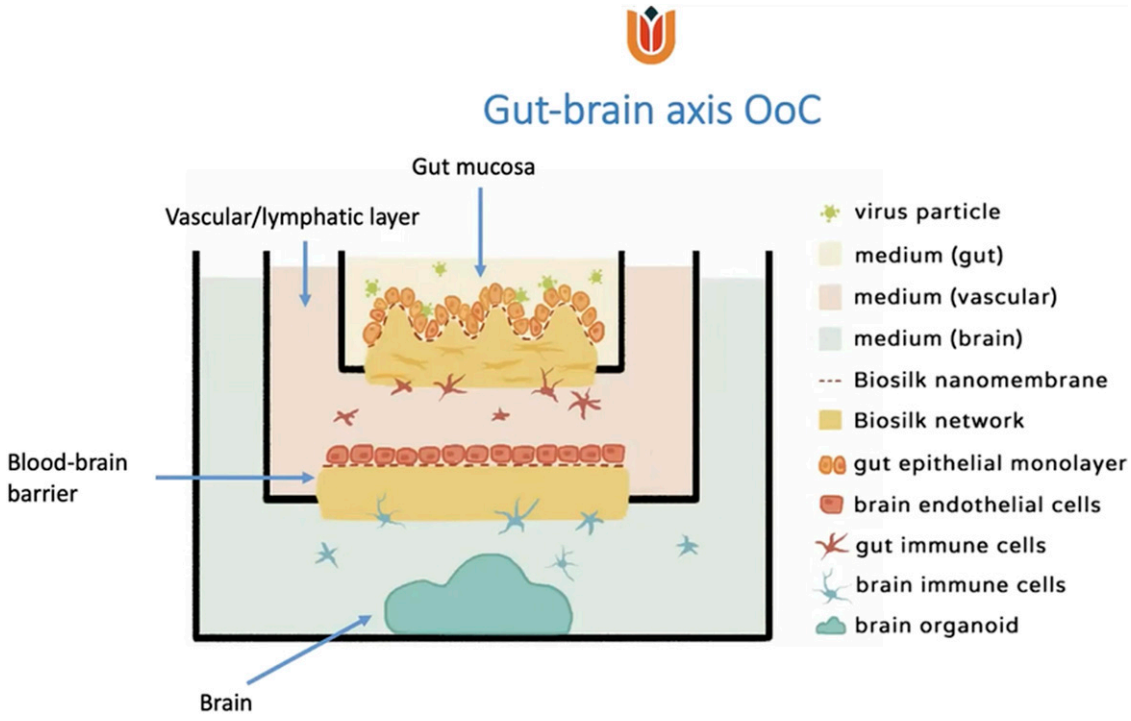

Figure 5. Gut–brain axis organ-on-chip (OoC).

pathogen interaction studies of viral infections in the respiratory tract (RT), while human gut and brain organoids facilitate human gastrointestinal (GI) tract and brain studies. The laboratory has established 3-D models of the human RT (by using HAE and lung organoids) and GI tract (with human gut organoids), in order to study SARS-CoV-2, picornavirus and CMV infections. Infection of these models with enterovirus A71, enterovirus D68,[83,84] human parechovirus CNS disease and SARS-CoV-2,[85] was also discussed. Professor Pajkrt concluded the talk by summarising the different uses of these models and highlighting the importance of the gut–brain axis organ-on-a-chip (see www.gutvibrations.org and Figure 5).

Choosing the right liver in vitro *model*

Dr Michael Johnson (CEO, Visikol, USA) presented his talk on choosing the right liver *in vitro* model, and discussed the advantages and disadvantages of various models. Visikol combines advanced imaging and cell culture tools with AI and image analysis, to provide pharma and biotech clients with the insights that they need to accelerate the development of their therapeutics through end-to-end projects. In executing an *in vitro* assay, their clients' goal is to mimic *in vivo* outcomes within an inexpensive and high-throughput assay. Over the last 10 years, major advancements have been made in the space of *in vitro* models, to better bridge the gap between *in vivo* results and *in vitro* data, such that the overall drug discovery pipeline has become more efficient. However, there is no single *in vitro* method that best meets the needs of every research question, as *in vitro* models are a trade-off between *in vivo* relevancy, cost and throughput.

Dr Johnson discussed the different liver models that are currently available — such as 2-D cell culture, simple spheroids, complex spheroids and *ex vivo* tissue slices — for hepatotoxicity/drug metabolism and pharmacokinetics (DMPK) studies and the investigation of various liver diseases. He talked about the HUREL® micro liver products developed by Visikol, which are customised for different species and demonstrate phenotypic stability and metabolic competency. The HepaRG/NPC (nonparenchymal cell) 3-D cell culture model exhibits (patho)physiologically relevant cell subtypes, including hepatocytes, liver sinusoidal endothelial cells, Kupffer cells, stellate cells and cholangiocytes, with characteristic features like glycogen storage, transporter protein expression and P450 enzyme expression. Visikol have also developed a primary human hepatocyte spheroid model for drug-induced liver injury (DILI) screening, to study indirect/immune-mediated DILI, non-alcoholic fatty liver disease (NAFLD), steatosis and NASH/fibrosis. Dr Johnson elaborated on a case study of precision-cut tissue slices as an alternative *in vitro* model for evaluating anti-fibrotic agents, and discussed the advantages and disadvantages of the three different approaches, namely: normal human tissue (*ex vivo* disease state

induction and therapeutic evaluation); diseased human tissue (*ex vivo* therapeutic evaluation); and the mouse disease model (*ex vivo* therapeutic evaluation). Normal human liver tissue slice assays tend to be lower throughput and more extensive, but are highly relevant; however, diseased human liver tissue slices do not last as long in culture as normal tissue slices.

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

Mr Tilo Weber (Animal Welfare Academy of the German Animal Welfare Federation, Germany) presented his talk on progress toward a truly animal-free laboratory through the use of alternatives to animal-derived reagents. The European *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.[86] However, this should also take into consideration the replacement of animal-derived laboratory reagents. To facilitate an optimal environment for cells to thrive *in vitro*, they must be held in homeostasis by maintaining medium supplementation, providing cell attachment factors, considering any necessary dish-coating materials and procedures, and also standardising the brand of the plasticware used. Unfortunately, many commonly used laboratory reagents and materials are still of animal origin. This causes not only immense ethical and animal welfare issues,[87,88] but also safety concerns resulting from the reduced reproducibility, reliability, transferability, and thus integrity, of any scientific data obtained.[89]

Among various animal-derived materials, such as medium supplements, cell attachment factors, antibodies and enzymes, Mr Weber discussed the production of fetal bovine serum (FBS) — from the procurement of the fetus to the final processing of the FBS. During the blood collection process, stunning or killing the fetus is not common and not compulsory — thus, animal suffering is neither excluded nor sufficiently alleviated.[87] In addition, living animal bodies are used as manufacturing devices, and animals can suffer from side effects and long-lasting consequences of these procedures (another example of this is growing cancer cells in mice to produce Matrigel®).[90] He highlighted a number of case studies exemplifying the transition to animal component-free cell culture,[91] and listed various replacements for animal-derived reagents — for example, the use of human platelet lysate (hPL) as a supplement for human mesenchymal stem/stromal cells, and the use of xeno-free chemically defined media.[91,92] Finally, Mr Weber provided guidelines to find,[93] as well as produce, optimised serum-free cell culture media,[94] and shared details of a practical workshop on replacing FBS.[95] He briefly touched on the replacements available for other animal-derived reagents and endotoxin/pyrogen tests,[91] hence setting the path to achieving a truly animal-free laboratory working environment, to the benefit of animals, scientists and patients.

Xeno-free bio-functional VitroGel system for 3-D cell culture and functional assays

Dr John Huang (CEO, TheWell Bioscience, USA) outlined the xeno-free bio-functional VitroGel® system for 3-D cell culture and functional assays. TheWell Bioscience is pioneering 3-D biomimicking platforms, driving advanced biomedicine and improving personalised medicine. The human body is a 3-D structure, and thus mimicking the human microenvironment is complicated. 3-D culture assays have become popular for studying complex systems, such as the tumour microenvironment, as they provide a more physiologically relevant cellular environment. Compared with traditional 2-D cell culture, which lacks tissue-specific architecture, the 3-D system allows insight into cell–cell interactions and communication. By using 3-D matrices containing the functional components of the extracellular matrix (ECM), researchers can even enhance the biological relevance of 3-D *in vitro* models. Traditionally, studies with 3-D matrices rely heavily on the animal-based Matrigel®. Despite the timely and innovative development of the Matrigel system, multiple complications have been encountered over the years, such as poor reproducibility, temperature sensitivity and the complexity of unknown proteins, which influence the test results.

To overcome these issues, TheWell Bioscience have developed the VitroGel hydrogel system, a new generation of animal origin-free functional hydrogel for advanced 3-D cell culture studies. The synthetic xeno-free bio functional hydrogel system offers many advantages over native ECM hydrogels, and allows room temperature operation with excellent batch-to-batch reproducibility. Two hydrogel formats are available: VitroGel ready-to-use hydrogel and VitroGel high concentration hydrogel kits. Dr Huang discussed five robust culture methods in which VitroGel can be used: 3-D cultures; 2-D hydrogel coating; static suspension culture; hydrogel cell bead; and animal injection. Besides closely mimicking the natural ECM, the VitroGel system has controllable properties in terms of mechanical strength, biological functional groups and degradability. This allows researchers to perform 3-D functional assays for in-depth examination of the various biochemical and biophysical factors involved in, for example, invasion, migration and angiogenesis. He also explained the VitroGel 3D culture process and presented the results of a viability assay of MCF7 cells cultured in a VitroGel hydrogel matrix for 35 days by using the Cyto3D® live–dead assay. Further, he also described a 3-D coculture that incorporates human

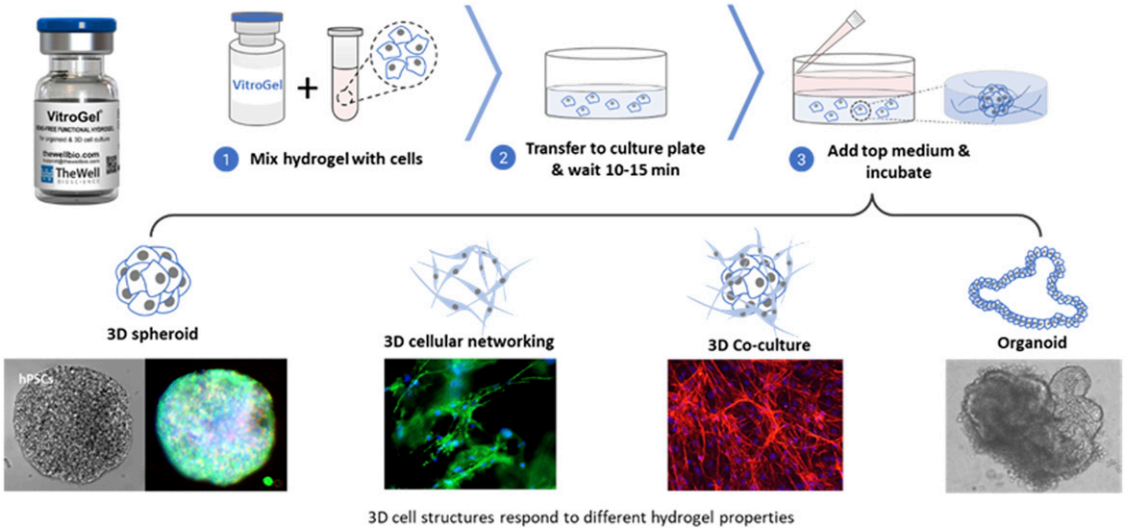

Figure 6. Examples of 3-D cell structures responding to different hydrogel properties.

breast cancer cells and normal human dermal fibroblasts, and explained how the properties of the hydrogel system can be controlled for conducting multiple types of assays.

The VitroGel angiogenesis assay kit gives full control for both 2-D and 3-D angiogenesis studies. Growth factors in the hydrogel play an important role in the process of tube formation, and the mechanical strength of the hydrogel system required for proper tube formation is highly specific. Dr Huang outlined a complete xeno-free organoid workflow, with the VitroGel STEM (for stem cell spheroid formation and differentiation) and VitroGel ORGANOID (for organoid formation), and compared it with the use of Matrigel. A synthetic hydrogel, VitroGel ORGANOID-3, improved immune cell–epithelial interactions in a coculture model of human gastric organoids and dendritic cells compared to the use of Matrigel. TheWell Bioscience also provide VitroINK, a ready-to-use, xeno-free functional bioink system for 3-D bioprinting projects (Figure 6).[96–98]

Organoids as a tool to explore the spectrum of human response to pathogens

Dr Lisiena Hysenaj (Scientist, Parvus Therapeutics Inc., USA), presented her lecture on the use of organoids as a tool to explore the spectrum of the human epithelial response to pathogens. She started her lecture by introducing the work done at the Roose Lab, which involved the banking of patient-derived lung organoids (PDOs).[99] The characterisation of lung epithelial cells by FACS showed that PDOs from different donors exhibit a distinct phenotype, and that PDOs of ciliated cells and the mucus-producing cell fraction correlate with the age of the donor. They found that with increasing age there is a decrease in organoids formed from mucus-producing cells. Furthermore, PDOs from different donors display different infection susceptibility to H1N1. Ancestral SARS coronavirus-2 (SARS-CoV-2), and subsequent variants of concern, have caused a global pandemic with a spectrum of disease variations linked to immune dysfunction, but the underpinnings of variation related to lung epithelium are largely unknown.

The Roose Lab showed that a large collection of lung organoids present distinct infection rates and epithelial cell responses for different donors, upon SARS-CoV-2 and influenza infection. Dr Hysenaj elaborated on what makes lung organoids susceptible to SARS-CoV-2. Single-cell RNAseq and spectral flow analyses of infected lung organoids identified tetraspanin 8 (TSPAN8) as a novel mediator and facilitator of SARS-CoV-2 — but not influenza — infection. TSPAN8 expression strongly correlates with SARS-CoV-2 infection rates. Reductionist HEK293T cell–pseudovirus approaches show that TSPAN8 enhances viral entry, but is independent of the spike–ACE2 interaction. In lung organoids, the delta and omicron variants of concern displayed lower overall infection rates in head-to-head comparisons with ancestral SARS-CoV-2. Detailed analyses of lung organoid epithelial cell composition and functional molecules revealed several nuances between variants of concern tested in lung organoids upon *in vitro* SARS-CoV-2 infection. All variants were able to infect many epithelial cell types, but shared the highest tropism for ciliated and goblet cells. Universal features of infected cells were ACE2, as well as TSPAN8, expression. Lastly, TSPAN8-blocking antibodies diminish SARS-CoV-2 infection, as shown in PDOs. Given the conserved use of TSPAN8 by all SARS-CoV-2 variants, TSPAN8 may be a novel avenue for COVID-19 therapy.[100]

Session 5: Carcinogenesis through spheroids

The study of spheroids *per se* can help in furthering our understanding of the processes associated with carcinogenesis and, in particular, metastasis.

The matrix is everywhere. It is all around us: Vindicating Morpheus through studies of cancer

Dr Ramray Bhat (Associate Professor, Department of Molecular Reproduction, Development and Genetics (MRDG), and BioSystems Science and Engineering (BSSE), Indian Institute of Science, Bangalore, India), presented his laboratory's research on invasive breast and ovarian cancers, wherein insightful transitions in the mesoscale morphogenetic behaviours of cancer cells have been observed, and linked to unique extracellular matrix (ECM) dynamics. Carcinogenesis can be conceptualised as a process by which principles governing the patterning of tissues into homeostatic multicellular architectures are violated.[101] Recent research on metastasis suggests that this is just part of the picture — as cancer cells travel through diverse ECM microenvironments, they adapt, organise and remodel their surroundings by evoking principles entrenched in morphogenesis.[102] Dr Bhat exemplified this notion by describing novel morphogenetic aspects of metastasis in breast[103] and ovarian cancers.[104] The time trajectory of metastasis can be broken into four stages: early migration from the primary focus; migration within fluid (circulatory) environments; intravasation into endothelial/mesothelial environments; and colonisation[105] — with morphogenetic features influencing each of these stages. In a study on breast cancer, he discussed how an interplay between computer and experimental models showed how intercellular heterogeneity in the surface expression of a glycan linkage, α2,6-linked sialic acid, resulted in a corresponding heterogeneity in cell–ECM adhesion. In turn, within confining 3-D ECM environments, this led to a corresponding heterogeneity in the behaviour of the cell population. Cells with higher expression formed a jammed and immotile stationary core, whereas those with lower expression formed an unjammed migratory front.[106] Such alterations in behaviours — likened to phase transitions in materials — are now thought to regulate several aspects of cancer behaviour.

Dr Bhat then focused on a second study, namely the migration of ovarian cancer spheroids through peritoneal fluid-like microenvironments.[107] His group has observed inter-spheroidal morphological heterogeneity within the ascites of patients, wherein spheroids resemble embryonic morulae (moruloid spheroids) and blastulae (blastuloid spheroids).[104] Patient-derived ovarian cancer cell lines, upon culture on low adhesion substrata, transition into moruloid spheroids and then blastuloid spheroids with correlative differences in basement membrane matrix formation and localisation, and Rho-based cytoskeletal modulation and polarisation. Moruloid spheroids adhere faster onto peritoneal surfaces. On the other hand, blastuloid spheroids, on account of their mechanically unique properties, are more resilient to stresses but adhere less frequently.[108] Dr Bhat discussed different approaches to attack these spheroids, employing omics-based analysis followed by drug screens.

Finally, he discussed the fourth stage of metastasis, i.e. colonisation, in which preliminary studies indicate non-intuitive behaviours behind collective migration that originates from settling cancer spheroids. He also briefly described some preliminary work on the distinct migratory behaviours of chemo-resistant cancer cells. He concluded that heterogeneity in morphological behaviours, both in cellular and multicellular cancer phenotypes, has deep consequences for metastasis. Unravelling and understanding such novel rules could help scientists and clinicians devise future management strategies.[102]

Special Session on '*In silico* strategies for preclinical research in oncology'

This special interactive session on '*In silico* strategies for preclinical research in oncology' was attended by scientists from some of the numerous complementary fields involved in the drug discovery and development journey — thus emphasising the importance of contributions from a wide range of scientific disciplines.

Three speakers, who represented different knowledge domains related to 'wet' and 'dry' laboratory activities in preclinical research, presented during the session (and are outlined below). The session was moderated by a mediator with experience in translational research, which set the context for an open discussion on the fast-evolving landscape of preclinical research and development, with an emphasis on the use of computational biology, AI/machine learning-driven applications and data science, to improve the efficiency of drug discovery and development.

Computational approaches for translational oncology

Dr Santosh Dixit (Chief Domain Expert, Healthcare and Life Sciences, Innovation Labs, Persistent Systems, India) set the context for the special session. In his opening remarks, Dr Dixit presented an overview of recent FDA initiatives for Three Rs compliance in preclinical research,

including the rapid adoption of *in vitro*, *ex vivo* and *in silico* models to reduce animal experimentation. He also reviewed the state-of-art in the field of oncology preclinical research, in reference to the FDA initiatives on Three Rs compliance. Lastly, Dr Dixit highlighted the need for collaborations between experimental biologists and computational scientists, to fast-track preclinical and translational research in oncology.

Dry lab challenges for wet lab scientists in preclinical oncology

Dr Mandar Kulkarni (CEO, VCR Park, India) emphasised the need for prioritisation of cancer research programmes based on cancer incidence in India. He explained the rationale behind choosing oral cancer for his teams' efforts in developing diagnostic and therapeutic research programmes. Accordingly, he discussed the potential pathways toward drug discovery and development for oral cancers unique to the Indian population. In these research roadmaps, he highlighted that discovery, translational and preclinical studies are expected to create a huge amount of research data. As a result, such translational research programmes should weigh in on data management and analytics capabilities, in addition to experimental biology readiness. He then discussed the need for identifying like-minded partners in the data science industry, who not only understand the newer concepts in data science and information technology, but also have a solid understanding of the oncology preclinical and translational research domain.

Data science applications to tissue microenvironment (TME) models

Mr Shreekanth Joshi (VP, Engineering, Healthcare & Life Sciences, Persistent Systems, India), elaborated on the machine learning applications for the design and development of microfluidics in organ-on-a-chip applications. He talked about the use of the data generated to improve the parameters of the experimental models, microfluidics, 3-D models and animal models. He also shed light on the various data processing opportunities and future directions.

Following these three presentations, Dr Dixit showed case studies in which preclinical research outcomes could potentially be improved by collaboration between experimental biologists and computational scientists. These include: a) applications of AI-driven knowledge graphs for rational generation of hypotheses to reduce animals in experimental validation; b) preclinical imaging of 3-D tumour spheroids and whole animals by using mass spectrometry imaging; c) multimodal data analysis by using MRI in animals for translational oncology applications; d) AI-based biomedical imaging for pharmacodynamics and tissue distribution studies of oncology drugs; e) the use of active learning/machine learning (AL/ML) for improving the outcomes of PBPK modelling; and f) the development of *in silico* whole animal models for pharmacokinetics/pharmacodynamics studies by using bio simulations.

Summary

This third international conference comprised six different, yet interrelated, scientific-themed sessions, including two keynote lectures and a special interactive session on *in silico* strategies for preclinical research in oncology, as part of a two-day programme. These sessions covered many of the issues associated with non-animal alternatives and the Three Rs in research, from the adoption of the Three Rs principles to the challenges involved in the various levels of their application, along with recommendations to tackle current obstacles. The aim of the conference, as for the previous two conferences in the series, was to bring together global researchers with a variety of expertise and interests, and to provide a platform to share and discuss their findings to promote practices aligned with the Three Rs principles. In addition, the conference presented the opportunity to network and find areas for collaboration among the speakers and the attendees from different fields. Although the speakers were from different scientific backgrounds, they are all working under the same umbrella of preclinical studies, *in vitro* disease modelling, and applying good practices according to the Three Rs principles.

Animal experimentation has been an integral part of the drug discovery and development journey, since it provides insight into drug mechanisms, pharmacokinetics and pharmacodynamics. However, animal models often fail to replicate the human situation faithfully, which is one of the biggest causes of drug failure as they move toward Phase II. Researchers across the globe are now considering the importance and relevance of the Three Rs concept in research and testing.

The Three Rs — *replacement*, *reduction* and *refinement* — can be applied to animal studies in various ways. The PREPARE and ARRIVE guidelines, presented by Prof. Adrian Smith, suggest the best practices that can be used to improve the scientific quality and animal welfare when planning animal studies, thus representing the application of *refinement*. In addition to scientific and ethical reasons to adopt the Three Rs, application of the principles is also supported by current legislative reforms, as mentioned earlier. Various human-based research and testing methods are currently in practice, including 3-D cultures, organ-on-chip/human-on-a-chip, organoids, imaging studies, biomarker studies, virtual humans and *in silico*/AI, as discussed in various talks at the conference. However, there are several barriers to the implementation of these *replacement* methods, as discussed by Dr Akhtar — for example,

miseducation, archaic policies, cultural bias and lack of funding. Possible solutions to the above-mentioned barriers are to educate the next generation, update policies, change the narrative, and increase investment and funding. Organisations such as the Center for Contemporary Sciences in the US, provide services to help to further the understanding and adoption of the Three Rs principles in animal experimentation and promote the use of alternatives, by educating the next generation and encouraging dialogue on the Three Rs principles.

In addition to the PREPARE and ARRIVE guidelines, another way of applying the *refinement* and *reduction* principles in animal experimentation is to ensure the reproducibility and validity of the experimental data by controlling various variables. One such variable that challenges the reproducibility of experimental data in preclinical research is the diet of the laboratory animals used, which can affect the data obtained. Furthermore, a rational approach to the Three Rs, while choosing conventional or specific pathogen-free (genetically and health-wise standardised) animals for preclinical studies, is essential to the validity of the data obtained.

As well as barriers such as miseducation, archaic policies, cultural bias and lack of funding to replace animal testing, there are also scientific barriers to the implementation of *replacement* methods, represented by systematic errors linked to R&D. There is a critical unmet need for *in vitro* models of human diseases for medical research purposes, or functional human tissues to support or replace injured tissues or organs *in vivo*. While many synthetic and natural non-human materials are already established for use in 2-D and 3-D applications, they still do not replicate the complex functions of the extracellular matrix in native, intact human tissue. To overcome systematic errors linked to R&D, THT Biomaterials are developing tools for a variety of applications and cell types, including 2-D and 3-D cell cultures, hydrogels, lab-on-a-chip, bioprinting, organoids and stem cells. Similarly, microphysiological systems (MPS) have proven to be a powerful tool for recreating human tissue-like and organ-like functions for research purposes, providing the basis for the establishment of qualified preclinical assays with improved predictive power. Established single-organ chips, as well as two-organ and four-organ chip solutions, are examples of such MPS, as well as a recently developed microfluidic multi-organ-chip platform that is the size of a microscope slide and integrates an on-chip micropump.

In vitro human-based experimental systems that can replicate human *in vivo* organ-specific functions are valuable preclinical tools for the assessment of human-specific drug properties. Dr Albert Li introduced and discussed novel human *in vitro* technologies developed by his company, such as the 999 Elite Cryopreserved Human Hepatocytes and MetMax Cryopreserved Human Hepatocytes, and their application in the evaluation of human drug properties during drug development. Another example of human-based experimental systems is the development of a 3-D printed mini bioreactor that mimics the *in vivo* arrangement of hepatocytes and the sinusoidal network, focusing on the zonation within the liver, as described by Dr Falguni Pati. New advanced platforms integrating native-like 3-D mechanical microenvironments with an unprecedented level of precision are now a reality, with the development of the beating organ-on-a-chip by Dr Paola Occhetta and her team. These represent more advanced and inclusive versions of the organ-on-a-chip concept, and show potential as preclinical tools for drug screening and disease modelling. In another example, Dr Prajakta Dandekar and her team developed different formats of non-animal alternative models for the preclinical evaluation of therapies for developmental retinal diseases.

Despite major progress in the field of *in vitro* models, animal studies remain a vital part of preclinical studies. Dr Manu Smriti Singh compared 2-D and 3-D spheroid-based mice tumour models developed through her research, and demonstrated that the use of 3-D spheroids instead of 2-D cancer cell cultures warrants better outcomes when screening anti-cancer therapies in clinical settings. Similarly, Dr Krishna Bhagavatula described the use of customisable humanised mice as a novel platform for preclinical research for infection and cancer studies, etc. In addition to drug testing prior to clinical studies, animals are also used for pyrogen testing. Dr Rohit Bisht from PETA recommended a novel human-relevant animal-free preclinical safety testing method for monoclonal antibodies directed against foreign targets.

In the field of disease models, Dr Dasja Pajkrt provided insight into the use of human organoids to study infectious diseases, such as SARS-CoV-2, picornavirus and human cytomegalovirus. Similarly, as explained by Dr Lisiena Hysenaj, organoids can also be used as a tool to explore the spectrum of human responses to pathogens. In addition to the use of spheroids as human-based alternatives to animal models, their study *per se* can also help to improve our understanding of carcinogenesis and, in particular, metastasis.

Over the last 10 years, major advancements have been made in the space of *in vitro* models to better bridge the gap between *in vivo* and *in vitro* data, such that the overall drug discovery pipeline has become more efficient. However, there is no single *in vitro* model that fully meets the needs of every research question, as *in vitro* models are a trade-off between *in vivo* relevancy, cost and throughput. Dr Michael Johnson talked about the many *in vitro* models that are available for the liver, and emphasised the importance of choosing the right liver *in vitro* model based on the disease being studied.

The complete replacement of animal studies during the drug discovery and development journey is still a long way off. However, we can start by replacing animal-based reagents. Mr Tilo Weber shed light on the progress toward a

truly animal-free laboratory through the use of alternatives to animal-derived reagents — for example, animal origin-free fetal bovine serum. Traditionally, studies with 3-D matrices relied heavily on the animal-based Matrigel. Despite the timely and innovative development of the Matrigel system, multiple issues have been encountered over the years, such as reproducibility, temperature sensitivity and the complexity of unknown proteins, which influence the test results. To overcome these issues, TheWell Bioscience developed the VitroGel® hydrogel system, a new generation of animal origin-free functional hydrogel for advanced 3-D cell culture studies and functional assays.

In the Special Session on '*In silico* strategies for preclinical research in oncology' scientists from a range of complementary fields involved in the drug discovery and development journey were brought together, to emphasise the importance of their individual contribution. Collaboration between 'dry' and 'wet' laboratory scientists is critical to promoting the effective and efficient application of the Three Rs principles at every step of the drug discovery and development process. Thus, we hope that all of the advances collectively made will push drug discovery and *in vitro* disease modelling closer toward a global paradigm shift.

Conclusions

To conclude, the barriers to adopting the Three Rs principles are being addressed globally at scientific, ethical and legislative levels. As highlighted, numerous human-derived *in vitro* models are available, at various levels of complexity, for use according to the research question, starting from 3-D cultures, organs-on-chips, beating organs-on-chips, etc. These models often employ the *replacement* and *reduction* principles of the Three Rs. Thus, considering all the advances in the field, it is time to collaborate in order to maximise our efforts toward:

- *reduction* of animal use in research;
- *refinement* of the animal experiments that are still undertaken; and
- full *replacement* within a completely animal-free laboratory, starting with the reagents used.

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Nikita Narayan Naik, Bharadwaja Vadloori and Suresh Poosala drafted the manuscript. All authors contributed to the content of the manuscript.

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