3rd 3R-Symposium

"Animal-free Methods in Pharmaceutical Research"

October 18th, 2024

Saarland University, Campus Saarbrücken – Aula A3 3

Organizers and Chairs

Dr. Marius Hittinger, Dr. Christina Körbel, Prof. Matthias Laschke,

Prof. Claus-Michael Lehr, Prof. Marc Schneider and Prof. Daniela Yildiz

Advisory Board

Dr. Sylvia Wagner, Prof. Martin Empting

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AGENDA:

9:00 Opening and Welcome Address

- **Sebastian Thul**, Secretary of State, Saarland Ministry for the Environment, Climate, Mobility, Agriculture and Consumer Protection
- **Prof. Dr. Robert Ernst,** Vice president for Research and Outreach, Saarland University

09:30 Keynote I (Chair: Prof. Lehr)

Prof. Nicole Teusch, Department of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Düsseldorf:
Tumor microenvironment on-chip model for precision drug discovery in pancreatic cancer

10:00 Short presentations I (Chair: Prof. Schneider)

- Dr. Yvonne Kohl, Fraunhofer Institute for Biomedical Engineering IBMT, Sulzbach:
  New Approach Methods for predictive toxicology: Are we on the right track? (10:00 – 10:15 a.m.)
- Dr. Maximiliane Wußmann, Fraunhofer Institute for Silicate Research ISC, Würzburg:
  Efficacy in Focus: In Vitro Barrier Models for Pharmacological Studies (10:15 – 10:30 a.m.)
- Dr. Sabrina Schnur/Verena Vogel, Department of Pharmaceutics and Pharmaceutical Technology, UdS/HTW, Saarbrücken:
  Development of a modular fluid system to investigate the permeation of drugs from BCS I and BCS III classes through an epithelial barrier to predict pharmacokinetics (10:30 – 10:45 a.m.)
- Simone Lichtner, PharmBioTec gGmbH, Schiffweiler:
  SaAr in vitro: Advancing Drug Safety and Efficacy with in vitro Models and High-Throughput Proteomics in inflammatory diseases (10:45 – 11:00 a.m.)

11:00 Group Photo and Coffee break

11:30 Keynote II (Chair: Dr. Hittinger)

Dr. Marta Venczel, Early Development, Sanofi, Frankfurt:
Advancing in vitro assays to substitute in vivo experiments

12:00 Short presentations II (Chair: Dr. Wagner)

- Aghiad Bali, Department Drug Delivery Across Biological Barriers, HIPS, Saarbrücken:
  3D-Bioprinting of bacterial biofilms for modeling chronic infections and investigating novel therapeutics and their delivery (12:00 – 12:15 p.m.)
- Dr. Anna M. Jötten, Ludwig-Maximilians-University | PHIO Scientific GmbH, München:
  Unveiling the Dynamics of Scratch Assays: Continuous, Label-Free Monitoring of Cell Migration Using Lensfree Imaging and AI (12:15 – 12:30 p.m.)
- Mariana S. Guedes, Institute of Experimental and Clinical Pharmacology and Toxicology, UdS, Homburg:
  A lentiviral reporter platform for live imaging screening of 3D human lung organoids (12:30 – 12:45 p.m.)
- Dr. Pia Empting, SCIVII-labs, Saarbrücken:
  3-R in regulatorischen Tierversuchen - Quo Vadis? (12:45 – 1:00 p.m.)

13:00 Poster Session & Finger Food

14:00 Keynote III (Chair's: Prof. Laschke/ Prof. Yildiz)

Prof. Stephanie Krämer, Laboratory Animal Science and Animal Welfare & 3R-Centre, Justus-Liebig University, Giessen:
Wo stehen wir in der biomedizinischen Forschung und welche Rolle spielt darin noch das 3R-Konzept?

14:30 Short presentations III (Chair's: Prof. Laschke/ Prof. Yildiz)

- Dr. Charline Sommer, Fraunhofer Institute for Toxicology and Experimental Medicine, ITEM, Hannover:
  Using irAOPs to Uncover Immunotoxicity Mechanisms and Refine Experimental Approaches (2:30 – 2:45 p.m.)
- Nicolas Freche, Department of Radiation Therapy, Saarland University Hospital, Homburg:
  Isolated skin irradiation for minimal radiation exposure of other organs and tissues (2:45 – 3:00 p.m.)
- Dr. med. Jan M. Federspiel, Institute for Legal Medicine, UdS, Homburg:
  Postmortem retrograde-dye perfusion of the human heart – First steps in body donors towards a postmortem functional assessment of the heart (3:00 – 3:15 p.m.)

15:15 Adjourn

Abstract Handout

Session I

Abstract 1 (Kohl *et al.*)

NAMs for predictive toxicology: Are we on the right track?

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The Food and Drug Administration's (FDA) Predictive Toxicology roadmap published in 2017 lists "new methodologies and technologies to expand FDA's toxicology predictive capabilities and to potentially reduce the use of animal testing" [1]. The FDA Modernization Act 2.0 in 2022 removed the need to perform animal testing for any new drug development. These regulatory amendments offer the chance to replace animal testing in drug development with appropriately validated methods of predictive toxicology [2].
Predictive Toxicology uses New Approach Methodologies (NAMs) to assess the potential toxic effects of a chemical or drug on living organisms including humans, as well as to estimate potential risks associated with exposure to chemicals, drugs and other substances [2]. While NAMs are often associated with in vitro testing using human cells or tissues and establishing adverse outcome pathways (AOPs) for human disease outcomes, they cover a more wider range of methods, including quantitative structure–activity relationship predictions, high-throughput screening assays, omics applications, microphysiological systems and artificial intelligence [3].
A large number of promising NAMs resulted from many research projects during the last years. While some success has been achieved, many of the NAM approaches have not been formally validated. At the current status, several obstacles hamper a broader application of NAMs in current safety assessment. The limitations in addressing repeated dose toxicity, particularly chronic toxicity, represent a major challenge for the introduction of NAMs in a broader context. In addition, issues of predictability, reproducibility need to be clarified and the regulatory framework adapted to NAMs [2].
With an increasing need to incorporate NAMs in safety assessment and the need to find alternatives to animal testing, this presentation discusses the developments of recent years and highlights advantages and disadvantages. Taking the Horizon 2020 project SSbD4CheM (Safe and Sustainable by Design framework for the next generation of Chemicals and Materials) [4] as an example, in vitro models, as e.g. models based on human induced pluripotent stem cells, and tools, as different microfluidic approaches, developed at Fraunhofer IBMT are presented that are applicable for NAM-based approaches for safety assessment. The SSbD4CheM team develops a comprehensive Safe and Sustainable by Design (SSbD) framework that uses new science-based approaches, to identify potential hazards and risks, and innovative technologies to support the design of safer and more sustainable products and processes. As SSbD4CheM partner, Fraunhofer IBMT develops innovative approaches to better simulate the physiology of the human organism in vitro and thus achieve a more meaningful prediction of the safety assessment.

Acknowledgments: This project has received funding from the European Union's Horizon Europe research and innovation programme under grant agreement n° 101138475. UK participants in SSbD4CheM project are supported by UKRI. CH participants in SSbD4CheM project receive funding from the Swiss State Secretariat for Education, Research and Innovation (SERI). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union. Neither the European Union nor the granting authority can be held responsible for them.

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Abstract 2 (Vogel *et al.*)

Development of a modular fluid system to investigate the permeation of drugs from BCS I and BCS III classes through an epithelial barrier to predict pharmacokinetics

Verena Vogel[1,2], Sabrina Schnur[2], Sophie-Marie Klär[2], Dietmar Brück[1], Benedikt Faupel[1], Ralf-Kilian Zäh[1], Marc Schneider[2], Marius Hittinger[2,3,4]

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The process of developing new drug formulations is still based on pharmacokinetic data generated by animal studies however ethical concerns are driving the need for alternative methods and models. This study presents the development of a modular lab-on-desk system designed to reduce the use of animal experiments. The system is composed of multiple modules, each representing a LADME (Liberation, Absorption, Distribution, Metabolism, and Excretion) process or organ function. These modules can be independently operated or flexibly combined. Certain modules incorporate biological barriers, such as Caco-2 cells, to model the gastrointestinal tract, offering a platform to investigate the permeation of drugs through an epithelial barrier. A central compartment module acts as the core of the system and can be individually expanded to allow the combinations of modules and therefore LADME processes. Additionally, a module is designed to represent the liberation process of oral dosage forms. This module simulates the release of drugs from tablets into a liquid medium. Each module was custom-designed and manufactured using 3D printing techniques. A customized pump system enables the transport of media and substances between modules to enable inter-module communication.
For evaluating the fluidic platform, well-known drugs were selected representing different biopharmaceutical classes based on the Biopharmaceutical Classification System (BCS). Regarding this, the permeation of caffeine (BCS I) and acyclovir (BCS III) under dynamic conditions using the lab-on-desk system were investigated. As reference, transport studies under static conditions, using a classical Transwell® setup, were performed. The barrier integrity of the cells was monitored by measuring the transepithelial electrical resistance (TEER). The study investigates the initial phase of the oral administration route.
The apparent permeability coefficient (P_{app}) for acyclovir was observed to be 4.23×10^{-7} cm/s under dynamic conditions and 5.21×10^{-7} cm/s in static conditions (control). In contrast, caffeine demonstrated significantly faster permeation, with a P_{app} of 1×10^{-5} cm/s under dynamic conditions and 1.5×10^{-5} cm/s under static conditions (control). TEER measurements performed before and after the transport studies asses the barrier integrity. Initial TEER values of 1125 $\Omega\cdot cm^2$ (n=4) for the dynamic setup and 1102 $\Omega\, cm^2$ (n=4) for the static setup indicated an intact barrier. Post-experiment TEER values of 947 $\Omega\cdot cm^2$ (n=4) for the dynamic setup and 1100 $\Omega\cdot cm^2$ (n=4) for the static setup confirmed maintained barrier integrity throughout the study.
The developed system successfully combines modules and enables the transport of substances between them and provides a platform for investigating the permeation of substances in a more physiological environment. This enables a categorization of substances based on solubility and their intestinal permeability. Future steps include integrating an automated release for oral dosage forms and implementing a customized online TEER measurement to continuously monitor barrier integrity during experiments. Additionally, the study will focus on the pulmonary administration route, including Calu-3 cells. The more physiological environment in the LADME-on-desk system has the potential to improve *in vitro-in vivo* correlations, leading to a more reliable prediction of human pharmacokinetics and a tool for diverse applications in research.

Acknowledgments:

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Abstract 3 (Lichtner *et al.*)

SaAr *in vitro*: Advancing Drug Safety and Efficacy with in vitro Models and High-Throughput Proteomics in inflammatory diseases

Simone Lichtner[1]; Johanna Frey[1]; Janina Osti[1]; Selina Dannheimer[1]; Marc Schneider[2]; Marius Hittinger[1,2,3]

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The project "SaAr-in-vitro" (**s**chnelle **a**nwendungsorientierte **A**nalyse **r**obuster *in vitro* Systeme) focuses on research of inflammatory diseases such as asthma, COPD, Crohn's disease, psoriasis and inflammatory bowel disease (IBD). In the research project, the use of mass spectrometry in combination with in vitro cell culture models is used to enable the measurement of various inflammatory signals from skin, intestinal, and lung models, promoting safe, rapid, and reliable testing of drugs with high predictivity for human data. The recently established proteomics workflow has already shown promising results in first studies in LPS-stimulated and non-stimulated monocytes-derived macrophages (MDM). A total of 600 proteins were measured in MDM lysate and 245 proteins in MDM supernatant samples.
The analysis of the lysate and the supernatant showed that the majority of proteins were found exclusively in one of the two groups. The proteins detected in both groups were oppositely regulated, proving that the proteins in the supernatant are those secreted by the cells. Furthermore, changes in protein expression between unstimulated (negative control) and inflamed (positive control) MDM were confirmed. The in-depth analysis of the proteome revealed that the positive controls of lysates and supernatants had a significantly higher number of proteins, compared to the negative controls. 86 proteins in the lysate and 21 proteins in the supernatant were identified as being uniquely expressed in the positive controls and are directly assigned to macrophages' response to inflammation. After biomarkers that can be measured reliably and reproducibly have been identified based on the measurements, the test systems will be validated by treating stimulated cells with known anti-inflammatory drugs, to evaluate drug safety and efficacy. First results in drug-treated MDM already confirmed the drugs' efficacy by comparing the proteome of inflamed and treated samples. A downregulation of of pro-inflammatory proteins was clearly detected.
Within the next milestones the current insights are extended to co-culture models to improve predictability of the models of inflammatory diseases.
Ultimately, "SaAr in vitro" advances targeted drug development and early hazard detection, and additionally promotes the 3R principles, offering ethical and effective alternatives to animal testing.

Acknowledgments: The presented work was financially supported by the EFRE project "

Session II

Abstract 4 (Bali *et al.*)

3D-Bioprinting of bacterial biofilms for modeling chronic infections and investigating novel therapeutics and their delivery

Bali A.[1,2], Latta L.[1], Bankar N.[1,2], Aliyazdi S.[1,2], Titz A.[1,3], Bals R.[1,4], Yildrim T.[1], Loretz B.[1], Schneider-Daum N.[1], Lehr C-M. [1,2]

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Biofilm-forming bacteria can be up to 1000 fold more tolerable to antibiotic treatments than their planktonic counterparts [1] , which necessitates the urge to find novel therapeutic approaches. Novel developed drug formulations must be tested for safety and efficacy before subsequent clinical studies may be considered. The current standard to test these novel antimicrobials are animal models which are, however, ethically problematic and moreover limited by anatomic, genetic and immunological differences to humans. Current *in vitro* models of bacterial biofilms do not allow addressing any host-pathogen interactions or show drawbacks in terms of reproducibility and design.

Expanding on recently reported pipetting of biofilm microclusters [2], we here report a novel approach using 3D-bioprinting to generate an improved human-based *in vitro* infection model. First, we developed a universal bioink for bacterial cultivation based on agarose-alginate. The developed bioink proved to be suitable to print biofilms of *P. aeruginosa* and *S. aureus*, the main pathogens inhabiting the lungs of cystic fibrosis patients [3]. Biofilm properties were maintained after printing as shown via microscopy techniques as well as in antibiotic susceptibility assays.

Secondly, we adapted the technique to generate a high-throughput platform for testing anti infectives and drug candidates for their MIC and MBEC values in a stimulaneous read-out photometrically. This was further supported by generating a model to investigate the permeation of nanoparticles and drug molecules through biofilms of different bacterial strains by implementing the 3D-bioprinting technology to introduce evenly distributed layers of bacterial biofilms on transwell inserts.

Finally, the established blank bioink was printed on epithelial cells to exclude any negative impact on cell viability and barrier integrity of the epithelial cells. Furthermore, thin biofilm constructs of *P. aeruginosa* were printed on confluent layers of human bronchial epithelial cells (Calu-3) grown at Air-liquid-Interface to establish a complex *in vitro* model of chronic lung infection. Complex *in vitro* models comprising bacteria and human cells may allow to generate readouts for both the pathogens (e.g. killing, virulence factors) as well as for the epithelial cells (e.g. vitality, barrier properties) in parallel. The model is currently being further validated as a potential alternative to animal experiments for preclinical testing of novel antiinfectives.

Acknowledgments: The SET Foundation (Frankfurt, Germany) is thanked for financial support.

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Abstract 5 (Jötten *et al.*)

Unveiling the Dynamics of Scratch Assays: Continuous, Label-Free Monitoring of Cell Migration Using Lensfree Imaging and AI

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Scratch assays are essential for studying cell migration and wound healing in cellular biology. Traditional methods, relying on intermittent measurements and labeling, can disrupt cellular behavior. This study introduces a novel approach utilizing continuous, automated, lensfree imaging and analysis within CO2 incubators, providing detailed and accurate analysis of scratch assay dynamics.

We developed an advanced system for non-invasive, label-free imaging to continuously monitor the closure of scratch "wounds" in 2D cell cultures. This methodology enables real-time data acquisition, significantly enhancing the temporal resolution of cell movement observations compared to manual acquisition (every 30 minutes vs. every 8-12 hours). By tracking closure speed and direct cell movements without external markers, we gain a comprehensive understanding of cell dynamics.

Our findings demonstrate that this continuous monitoring system not only accurately measures wound closure rates but also distinguishes between active directed migration and proliferation-driven movements. This distinction is critical, as it provides a reliable indicator for differentiating these mechanisms in a label-free environment. The ability to discern these processes opens new avenues for understanding cell behavior in various biological contexts, including cancer metastasis and tissue regeneration.

Overall, this study underscores the potential of automated, continuous lensfree imaging systems in CO2 incubators to revolutionize cell migration assay analysis. By eliminating disruptive labeling and enhancing data resolution, our approach significantly advances cellular dynamics research, providing nuanced insights into cell behavior and movement mechanisms.

Abstract 6 (Guedes *et al.*)

A lentiviral reporter platform for live imaging screening of 3D human lung organoids

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Over the last decade, research efforts towards developing sustainable human models have been undertaken to increase the likelihood of success of new drugs taken into clinical trial phase, while circumventing animal experimentation.
In line with this goal, we developed a lentiviral reporter system to tackle differentiation, mucus production and drug screening in human-derived organoids. We designed fluorescently-labelled lentiviruses targeting gene promoters related to ciliation and mucus production, which were used to transduce apical-out organoids and investigated changes overtime in an automated live imaging system. During differentiation, organoids were stimulated with cytokines[1], bacterial proteins[2] and inhibitors[3,4], to test the reproducibility of the lentiviral system to accurately mimic cell and tissue physiology.
Our results reveal an increase in the promoter gene expression of markers for ciliated cells and mucus producing cells over time. Stimulation of the organoids with IL-13 significantly impairs ciliated gene expression, while pre-treatment with JAK-STAT and NOTCH pathway inhibitors rescues this phenotype. Interestingly, the lentiviral reporters were unable to detect any changes in the most abundant gene markers of mucus producing cells upon stimulation with several pro inflammatory compounds. Proteomic and transcriptomic analysis correlate and support these findings, and further allowed the identification of other potential markers of interest such as ALOX15. This is a gene that encodes a member of the lipoxygenase family which is highly expressed in bronchial epithelial cells and has been associated with the increase of MUC5AC in asthma, both in-vitro and ex-vivo[5,6]. Preliminary results on the lentiviral system indicate a similar pattern of overexpression of ALOX15 promoter activity with IL-13 stimulation and attenuation with JAK-STAT inhibitor compound.
Taken together, our results show that this novel system represents a robust tool for studying the complexity of the human lung epithelium and present a platform for screening of drugs and novel compounds in a high-throughput manner. Additionally, the versatility of lentiviral manipulation means that our model might have broader applications beyond the human lung and can be established for other organs and disease models.

Acknowledgments: BMBF 3D-REPLACE (16LW0140K)

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Poster Session

Abstract 7 (Desai *et al.*)

Refinement of current compound-eye interaction evaluation assays through standardized in vitro human cornea

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Eye drops are the most frequent and convenient way to treat the eye. For eye drops to reach the anterior part of the eye, they need to pass through the cornea, the main anatomical barrier for eye drop absorption. The cornea has a complex and layered structure, each layer with different properties and functions. Currently, the gold standard method to assess ocular toxicity of drugs is the Draize test in rabbits. There is only one method to test both absorption and permeation of ocular drugs. This method is based on surplus of cow/chick eyes from the meat industry. However, differences in the structure, thickness, composition, etc. between these animals and humans, and the variability in the obtention of quality eyes (similar age, size) make this method less predictive and less relevant [1]. In this context, laboratory-made cornea could improve the way we assess ocular drug absorption and permeation, as it could recreate the human corneal structure in a more standardized way. Despite these promising results, the fabrication of human cornea in the laboratory only allows for the assessment of a few drugs at a time and therefore standardization and validation are difficult [2]. This project aims to engineer a more relevant model of the human cornea in which absorption, permeation and toxicity studies can be carried out in vitro and in a high-throughput manner [3]. Until now, we have optimized culture parameters to differentiate human primary corneal epithelial cells at the air-liquid interface and our results also show that human primary keratocytes can be encapsulated in collagen hydrogels with high efficiency. Future work will deal with miniaturization of the optimized layers to fabricate the first human cornea-on-chip that recapitulates all three cellular layers to increase the throughput of the system.

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Abstract 8 (Mohizin *et al.*)

3D-printed microfluidic devices and cell-laden microgels: Potential opportunity to a novel type of organ-on-a-chip platform

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Integration of microscale hydrogel (termed as the microgel) components in microfluidic devices have recently been an essential engineering element for developing advanced biochips. This is mainly because cell-laden microgels are attracting great interests in the areas of tissue engineering and drug development, due to their prominent physiological functionality and compatibility to 3D cell culture techniques [1]. In microphysiological systems, integration of such miniaturized components allows the creation of on-chip organ-mimics and in vivo-like microenvironments [2]. Furthermore, the advent of 3D printing technology enabled rapid fabrication of microfluidic devices with complex channel architectures, on the basis of digital design and automatized manufacturing frameworks.

In this presentation, a few works of our group will be presented as examples of the modeling and fabrication of extracellular matrix (ECM)-mimicking microgel matrices [3-6]. For the purpose of regenerating artificial 3D tissues in dynamic culture systems, we introduce physicochemical fabrication methods to encapsulate mesenchymal stem cells and human liver cell lines in covalently crosslinked gelatin microgel spheres. By utilizing 3D-printed microfluidic devices, the microgel generation processes can be performed with various experimental conditions to control the monodispersity, matrix elasticity, cell seeding density, and scale factor of the microgels. We discuss the future direction of our research to develop novel technologies for alternative animal testing, such as microgel-based organ-on-a-chip and high-throughput in vitro toxicity screening. For those purposes, the 3D printing-based microdevice fabrication technique can be used for facile implementation of microfluidic chip structures, in which cell-embedded ECM-like microgels are integrated as organotypic 3D-cell compartments. The on-chip and ex chip evaluation methods of cytotoxicity after exposure to xenobiotic compounds are to be developed accordingly. Our work aims to develop modular organ-on-a-chip tools with user-friendly interface, which are adapted in the fields of pharmaceutical sciences and environmental toxicology.

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Abstract 9 (Vogel *et al.*)

Development of a multisensory system to monitor crucial parameters during transport studies in a multi modular fluid system

Verena Vogel[1,2], Sabrina Schnur[2], Ralf-Kilian Zäh[1], Marius Hittinger[2,3,4], Dietmar Brück[1], Benedikt Faupel[1], Marc Schneider[2]

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Incorporating sensor technology can greatly enhance the advancement of in vitro systems aimed to reduce animal studies in research. We have developed a lab-on-desk system with multiple modules that could offer an alternative to animal studies for predicting human pharmacokinetics. Some of these modules include biological barriers that simulate the epithelium of the gastrointestinal tract. To ensure cell culture functionality and improve the comparability of independent experiments, we develop a sensor system that monitors crucial parameters, such as temperature, pH levels, and transepithelial electrical resistance (TEER) during transport studies.

The system is controlled by an Arduino Mega 2560 microcontroller, which receives, records, and stores data on an SD card shield. Data can also be displayed in real time on a liquid crystal display (LCD). The setup includes a PT-1000 temperature sensor, whose signal is amplified to monitor environmental temperature. A liquid pH value detection module connects to the microcontroller via a BNC interface. The pH sensor setup was validated using reference standard buffers demonstrating a pH of 4.06 for a pH 4 buffer solution and 6.97 for a pH 7 buffered standard solution. To monitor TEER, which provides insight into cell barrier integrity, we developed a custom circuit connected to electrodes, consisting of 316L stainless steel filaments, on both the apical and basolateral sides of a epithelial barrier. A pulsed DC signal with a frequency of 490 Hz, generated by the controller, passes through the circuit that includes an exchangeable ohmic resistor and the sample of interest. The TEER measurement setup was optimized by investigating parameters such as signal duration and applied voltage. A 100 Ω resistor used as a control yielded a measurement of 104 Ω, demonstrating the accuracy of the setup and measurement process. Resistance measurements performed on a classical cell culture insert showed 950 Ω for a blank membrane filled with HBSS buffer and 1400 Ω for a Transwell® filled with agarose gel as a barrier model. To transfer the setup validation measurements on Caco-2 on a Transwell® membrane were performed. TEER measurements with a commercially available EVOM® device before and after using the custom TEER setup should ensure the barrier integrity was not affected by the system itself. The results indicated that the duration and frequency of the pulsed signal had a significant impact. A 60s DC signal harmed the Caco-2 barrier (TEER < 500 Ω/cm²), whereas a 2-second measurement interval with a 5-second pause maintained barrier function (TEER > 500 Ω/cm²).

The developed monitoring system effectively tracks temperature and pH levels. A customized TEER measurement system was validated through initial tests. Sensor data can be stored and displayed in real-time. Further steps will be the integration of a flow rate sensor. Future integration of this sensor system into the lab-on-desk platform during transport studies is planned. Overall, this monitoring system could enhance the lab-on-desk system's functionality, improve the comparability of experiments, and strengthen the in vitro-in vivo correlations, potentially leading to more reliable predictions of human pharmacokinetics.

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Abstract 10 (Brand *et al.*)

IL-13 drives a mucin phenotype: insights from a human apical out airway organoid model

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Abstract

Chronic lung diseases (e.g. chronic obstructive pulmonary disease, cystic fibrosis) are incurable. Epithelial senescence, a state of dysfunctional cell cycle arrest, contributes to the progression of such diseases. We differentiated basal cells to airway (bronchial) organoids. Single cell sequencing showed that the organoids were composed of (transitional) basal, secretory, goblet, deuterosomal and ciliated cells. In addition, we identified a cluster, here defined as senescent cells, with high expression for the common senescence markers CDKN1A (p21), CDKN2A (p16), TIMP2, and GDF15. Senescent cells were marked by an increased activity of the BATF and PRDM1 regulons. Pathway enrichment analysis and analysis of gene set activities showed enrichment of the cellular senescence pathway in the senescent cell cluster. Transitional basal cells and secretory cells showed velocity towards senescent cells. Treatment with the senolytic drug quercitin resulted in a reduced proportion of senescent cells, whereas doxorubicin treatment increased the proportion of goblet cells with increased expression of genes associated with detoxification (e.g. CYP1A1, ALDH13A). Quercetin antagonized doxorubicin-induced activity of senescence associated galactosidase. Further studies need to clarify to what extent such senescent cells are present in the airways of patients with chronic lung diseases and whether they affect the differentiation of the airway epithelium.

Abstract 11 (Brand *et al.*)

Identification of senescent-like cells in airway organoids using single cell sequencing

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Abstract
Chronic lung diseases (e.g. chronic obstructive pulmonary disease, cystic fibrosis) are incurable. Epithelial senescence, a state of dysfunctional cell cycle arrest, contributes to the progression of such diseases. We differentiated basal cells to airway (bronchial) organoids. Single cell sequencing showed that the organoids were composed of (transitional) basal, secretory, goblet, deuterosomal and ciliated cells. In addition, we identified a cluster, here defined as senescent cells, with high expression for the common senescence markers CDKN1A (p21), CDKN2A (p16), TIMP2, and GDF15. Senescent cells were marked by an increased activity of the BATF and PRDM1 regulons. Pathway enrichment analysis and analysis of gene set activities showed enrichment of the cellular senescence pathway in the senescent cell cluster. Transitional basal cells and secretory cells showed velocity towards senescent cells. Treatment with the senolytic drug quercitin resulted in a reduced proportion of senescent cells, whereas doxorubicin treatment increased the proportion of goblet cells with increased expression of genes associated with detoxification (e.g. CYP1A1, ALDH13A). Quercetin antagonized doxorubicin-induced activity of senescence associated galactosidase. Further studies need to clarify to what extent such senescent cells are present in the airways of patients with chronic lung diseases and whether they affect the differentiation of the airway epithelium.

Abstract 12 (Dehne *et al.*)

DEVELOPMENT OF A MARKET-READY QUALITY CONTROL SYSTEM FOR DETERMINING THE ELECTRICAL RESISTANCE FROM HUMAN SKIN SAMPLES – SkinTER

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The permeation of drugs from topical products through skin can be investigated via different ways. A standardized and well described method is the in vitro permeation test (IVPT) using human skin samples. Skin barrier integrity tests are highly recommended before using skin for *in vitro* permeation tests. This allows comparability of different samples and can prevent unnecessary repetition as well as the increase in project costs. In this project the focus is the development of a market-ready quality control system, named SkinTER, based on the electrical resistance, which allows a fast, simple and non-destructive way to measure skin samples. For the acceptance criteria evaluation an experimental study was conducted using skin of different donors and skin types with varied damaging intensities. The IVPT shows a successful correlation between the flux of the tracer molecule caffeine and the TER values measured before starting the permeation. These results can be used to define a cut off value for an intact skin barrier. Furthermore, corrosion testing was performed based on the OECD 430 [1] test guideline. A preliminary experiment with a small selection of corrosive and non-corrosive chemicals shows that the reported *in vivo* data in literature corresponds to the collected *in vitro* data. A difference in the sub-categories 1A and 1B/C was not apparent in the data collected so far. However, due to the collected data the SkinTER device is a promising tool to ensure comparability between different samples and to examine the integrity of the skin barrier.

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Abstract 13 (Osti *et al.*)

Development and standardization of a nasal and pharyngeal model for analyzing pharmaceutical deposition and surface coverage in the upper respiratory tract

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Due to the rising number of infectious diseases that are transmitted via the respiratory tract, the nose is playing an increasingly important role. Pathogens such as SARS-CoV-2 or influenza viruses can initially infect the nasal epithelium and spread to the lungs. From a therapeutic or preventive perspective, the nasal route offers a chance for preventing the entry of such pathogens as well as disease treatment. The selection of the correct device and ensuring a sufficient distribution of the nasal spray within nasal cavities are the bases of a successful nasal application. We aim for developing a sufficient human relevant nasal model for testing the spray behavior and its deposition and distribution in the inner nose.

The system is based on transparent 3D Koken® models from Erler Fischer [1]. Our setup consists of a box including the 3D-model, a camera, a holder for the nasal spray and a fluorescence light source. Parameters such as spray angle, the design of the nasal spray head and the influence of the formulation composition are currently being investigated.

The setup was optimized with focus on avoiding light scattering artifacts, selecting a suitable camera filter, camera settings and distance from camera to object. First experiments were conducted with aqueous buffer containing different concentrations of sodium fluorescein. Beside the successful physical setup, we could observe a strict dependency on spray angle which is directly linked to patient compliance. In the next steps, the composition of different drug formulations, device-designs as well as spray distribution in different regions inside the nose will be in focus.

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Abstract 14 (Neu *et al.*)

The novel monoclonal human alveolar epithelial cell line "Arlo" for modeling the pulmonary air blood-barrier in vitro

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In vitro models of the deep lung are important tools for investigating the healthy and diseased state of the respiratory tract. The alveolar epithelium compromises type-1 and type-2 alveolar cells that form a tight barrier to protect the organism from pathogens and toxins. However, under pathophysiological conditions like infections with SARS-CoV-2, Influenza A, or bacteria this barrier can be compromised.

Disease-relevant in vitro models are a great alternative to overcome limitations and replace animal- and human ex vivo models to study the (patho)-physiology and its diseases and evaluate the safety and efficacy of drugs. While primary human alveolar epithelial cells (hAEpC) are often considered as the best representation of deep lung cells, cell lines offer promising alternatives due to their accessibility and reproducibility. Unfortunately, most available cell lines are unsuitable for many applications due to their lack of barrier properties. To address this need, we immortalized hAEpC and established the hAELVi cell line, which we then improved via single-cell printing to generate the monoclonal cell line "Arlo" [1, 2]. Arlo exhibits pronounced and reproducible barrier properties, strict monolayer formation, and TEER values of approximately 3000 Ω*cm².

To demonstrate the suitability of Arlo for modelling the air-blood-barrier, we are currently investigating Arlo in the following aspects:

1) Alveolar-specific drug permeability

2) Proteome and transcriptome analysis to identify lung-related barrier-determining factors

3) In vitro pulmonary edema model as screening tool for drug candidates

Overall, Arlo is a promising and versatile cellular tool for human disease modeling, drug efficacy testing, and fundamental research.

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Abstract 15 (Latta *et al.*)

Use of public transcriptional datasets for evaluating pulmonary in vitro models

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In vitro models of the lungs may be important tools for both pharmaceutical and infection research. However, evaluating and validating the translational predictively of complex in vitro models is challenging due to the limited availability of appropriate ex vivo/in vivo models. Pulmonary epithelial primary cells and cell lines may be used for Transwell®-based in vitro models, provided they express tight junctions to form a biological barrier. Complementary to established functional and morphological characteristics and consensus markers, we generated bulk RNA-Seq Data from primary cells and the recently established cell line Arlo [1] for comparison with publicly available Datasets of several (alveolar and bronchial) tight and leaky lung cell lines [2]. While Na+/K+ ATPase, and ENAC channel were characteristic for barrier-forming cells, identity markers for alveolar cell fate, such as NKX2-1 and HOPX, were not related to barrier properties or alveolar origin. Identifying correlations between expression patterns and alveolar-specific physiological function is an important task for future research. To investigate pathophysiological changes of the airway epithelium in the context of bacterial infection we generated transcription data after exposing the bronchial cell line Calu-3 cells to the P. aeruginosa virulence factor LASB. Differences to reported data of infected human airway epithelial cells [3] need to be further investigated, but support the value of transcriptional data for the validation of complex in vitro models.

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Abstract 16 (Clemenz *et al.*)

Glycocalyx and extracellular matrix manipulation for the treatment of malignant melanoma

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Malignant melanoma (MM) is one of the most aggressive and rapidly progressing tumors due to its high proliferative capacity, strong invasiveness, and significant metastatic potential. The incidence of MM is rising at an alarming rate compared to other human cancers, with genetic mutations in the BRAF gene present in approximately 50% of melanomas [1]. These mutations enhance ERK phosphorylation, leading to increased expression of CSPG4 (chondroitin sulfate proteoglycan 4), also known as NG2, which drives tumor proliferation and cell growth. In this context, the glycocalyx and the extracellular matrix (ECM) play important roles. The tumor cell surface is characterized by a dense glycocalyx composed of glycoproteins and proteoglycans. Proteoglycans have a core protein bound to glycosaminoglycans (GAGs), long, negatively charged, and hydrophilic polysaccharides [2]. In melanoma, the extracellular matrix (ECM) is often altered, contributing to tumor growth, invasion, and resistance to therapies. Alterations in ECM components, such as proteoglycans, influence cell signaling, enhance melanoma cell survival, and promote metastatic potential [3].
Vemurafenib is a targeted therapy used to treat malignant melanoma that harbors mutations in the BRAF gene, specifically the BRAF V600E mutation. BRAF is a serine/threonine kinase involved in the MAPK/ERK signaling pathway, which regulates cell growth and survival. In melanoma, mutations in BRAF lead to constant activation of the MAPK/ERK pathway, driving uncontrolled cell proliferation and tumor growth. However, despite initial effectiveness, many patients develop resistance to vemurafenib, often due to changes in the tumor microenvironment, such as increased matrix stiffness and collagen-rich structures [4].
Because of the role of CSPG4 in promoting melanoma aggressiveness, we explore in vitro models to target GAGs like CSPG4 in combination with established BRAF inhibitors in melanoma cell lines to potentially improve anti-cancer treatment strategies. Interestingly, we discovered a range of new genes that influence GAG levels in melanoma cells in a CRISPR/Cas-9-based genome wide screen [5]. In melanoma cell lines, we showed that the deletion of newly identified GAG-regulating genes leads to decreased levels of CSPG4, reducing the ability of melanoma cells to form colonies. This highlights the role of the glycocalyx in melanoma progression. Using the Sk-mel5 melanoma cell line, in vitro studies reveal that Vemurafenib exhibits an EC50 value in the low µM range. Furthermore, we showed that the cellular metabolic activity is affected. Our data show that in vitro models are valuable tools to further explore the matrix related drug resistance in MM.

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Abstract 17 (Paulitschke *et al.*)

Advancing Cell-Based Assay Reproducibility Through Lensfree Imaging and AI Integration

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The 3R (Replacement, Reduction, Refinement) principle is fundamental in minimizing animal use in life science research. However, achieving Replacement and Reduction is challenging due to the lack of viable alternatives. Traditional cell-based assays often lack temporal information and depend heavily on cell quality, impairing reproducibility. To address these issues, we present a novel, incubator-compatible approach for cell-based assays aligned with the 3R principles.
Our method utilizes lensfree imaging to exploit cells' inherent optical properties for non-invasive, label-free monitoring within a CO2 incubator. Additionally, our system integrates artificial intelligence (AI) for real-time analysis of key cell culture parameters, such as confluency, proliferation, and motility [1]. This integration allows for automated, objective data acquisition, enhancing assay standardization and result reproducibility.
In this study, two colon carcinoma cell lines with varying growth homogeneity were cultured and monitored using a lensless microscope setup under arsenic trioxide treatment. Results indicated that memory effects of prior subculturing influenced cell growth and multi-omics profiles, with higher homogeneity in earlier passages increasing the number of differentially expressed markers by up to 80% [2].
By providing continuous, label-free monitoring during incubation, our system offers a powerful tool for deeper insights into cell behavior. This method can quantify organoid growth dynamics and interactions in 3D cell models. Our approach not only adheres to the 3R principles by reducing reliance on animal models but also significantly improves data quality, control, and processing speed in cell-based research. This technology holds potential to address the reproducibility crisis and standardize future cell-based assays.

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Abstract 18 (Herrmann *et al.*)

Scaling up the production of human induced pluripotent stem cell-derived cardiomyocytes for the use in in vitro test systems

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Background – Up to date, the approval of new drugs for clinical trials still relies on animal testing. Besides ethical concerns, the generated data often cannot be simply transferred to humans, leading in the end to an approval of approximately only 10 % of all the drugs that have entered clinical trials [1]. The need for better pre-clinical test systems gave rise to in vitro models implementing primary human or induced pluripotent stem cell (iPSC)-derived organ-specific cells. Focusing on the heart, the in vitro culture of primary cardiomyocytes (CMs) bears difficulties like fast de-differentiation, lacking quantity and varying quality. This can be overcome by using standardized and quality controlled human iPSC-CMs (hiPSC-CMs), an in safety pharmacology already validated cell type [2,3], thereby paving the way to reduce animal models.

Results – For the generation of cardiac in vitro models, we were able to scale-up the production of hiPSC-derived CMs in a standardized and reproducible manner yielding two billion hiPSC-CMs within 8 days by using a stirred bioreactor system. The cells quality and a high purity were confirmed by a high expression level of up to 98% of cardiac troponin T. Subsequent 2D culture allowed the cells to develop towards a more mature state. Using these hiPSC-CMs, we succeeded in generating contracting 3D engineered heart tissues (EHTs) when co-cultured with primary human cardiac fibroblasts.

Conclusion – Scaling-up the generation of hiPSC-CMs allows the production of large and quality-controlled batches of hiPSC-CMs. These harbor a great potential for the setup of standardized cardiac in vitro models such as EHTs. We demonstrated the functionality of our hiPSC-CMs, making them an even better candidate for the setup of test systems for tissue engineering and large-scale drug screening.

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Abstract 19 (Fendahl, Kuhn *et al.*)

More research, less animals: perspectives on minimizing animal testing in preclinical phase

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The replacement of animal testing is an important issue not only in biomedical research, but also in drug development. Nevertheless, animal testing is still essential for testing of efficacy, safety and tolerability, especially in the preclinical phase. Although animal testing is still mandatory at some points, it is possible to minimize the number of animals during the preclinical phase.
This is one of the goals of MucosaTec GmbH. The start-up develops prophylactic and therapeutic drugs directed against pathogens of the respiratory tract. Especially first-in-class medications, for which no predecessors are known, and which possess a unique mechanism of action, face the challenge of needing comprehensive preclinical data regarding toxicity, immunogenicity, and pharmacokinetics. Given the novelty of the locally acting antiviral drug, AntiFlu-001, it is imperative to address various regulatory challenges to ensure its successful approval and integration into clinical practice. To avoid unnecessary animal testing, various regulatory authorities and experts are consulted to ensure exchange and facilitate a potential approval process that minimizes animal experiments. Additionally, the company aims to minimize the use of animal testing in the preclinical phase through targeted advance planning and characterization of its manufactured product by monitoring product quality using in-depth analyses based on ICH guidelines.
Furthermore, advanced *in-vitro* methods, including human cell lines, ex-vivo models and air-liquid (ALI) assays are used to determine efficacy and safety. One example is the use of Vitrocell Cloud Alpha 12, which enables a close replication of the human physiology with regards to the nebulization of the drug compound to human cell lines in ALI culture. By adopting this approach, the company enhances its commitment to reducing animal testing, while maintaining high standards of quality and reproducibility.

Session III

Abstract 20 (Sommer *et al.*)

Using irAOPs to Uncover Immunotoxicity Mechanisms and Refine Experimental Approaches

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Immune therapies revolutionize therapies, but their adverse effects remain a significant challenge. The pathophysiological mechanisms behind these immunotoxic reactions often are unresolved, and clarifying key cells and molecules is challenging due to the complexity of the immune system. One biologic of high therapeutic interest is interleukin-2 (IL-2), used to treat cancer and autoimmune diseases, but often associated with side effects such as skin rashes. To shed light on mechanisms driving IL-2-induced skin rashes, we developed a hypothetical immune-related adverse outcome pathway (irAOP) based on current literature. This irAOP identifies key cells and molecular drivers of toxicity, focusing specifically on essential components rather than mapping all potential immune interactions.

Our hypothesis, supported by existing studies, suggests that type 2 innate lymphoid cells (ILC2s) are predominantly activated in the skin of IL-2-treated patients, inducing a type 2-driven immune response resembling dermatitis. To investigate this, we conducted experiments in wildtype mice, injecting IL-2 directly into the skin. Importantly, we reduced animal usage by 50% by using the same animals' untreated skin as negative controls. Consistent with our irAOP, we observed a specific expansion of ILC2s in response to IL-2 injections, validating the relevance of these cells in the adverse outcome [1].

Additionally, the irAOP framework revealed potential alternative mechanisms not previously described such as IL-33 secretion driving positive feedback loops and differential regulation of IL-2 receptor (IL-2R) subunits. While IL-2Rα expression upon IL-2 stimulation has been well-documented, we focused on IL-2Rβ expression, crucial for IL-2 signaling, which had not been extensively studied. Our investigation using human peripheral blood mononuclear cells (PBMCs) demonstrated a cell-specific reduction in IL-2Rβ expression upon IL-2 stimulation. This was especially prominent in T cells and led to decreased IL-2R signaling predominantly in CD4+ regulatory T cells (Tregs) [2]. Reduced signaling in Tregs could contribute to immune imbalances in IL-2-treated patients, potentially leading to autoimmune like skin rashes, as observed in patients with IL-2Rβ deficiencies [3, 4].

Overall, IL-2-induced dermatitis appears to be dominated by type 2 immunity, potentially exacerbated by immune dysregulation due to reduced IL-2R signaling in Tregs. The irAOP approach thus offers a valuable tool for 1) simplifying the complexity of immunotoxicity by focusing on key cellular drivers, 2) identifying gaps in the literature, and 3) refining experimental designs. This strategy supports the principles of the 3Rs by reducing animal use and advancing the development of targeted in vitro models.

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Abstract 21 (Freche *et al.*)

Isolated skin irradiation for minimal radiation exposure of other organs and tissues

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Ionizing radiation is a common therapeutic modality for the treatment of various cancers. To study its effects and potential side effects, a mouse model has been developed. In previous studies, mice were subjected to either whole body irradiation or localized radiation to the skin, with dosimetry modeled via CT scans [1]. However, radiation exposure to surrounding tissues can lead to unintended tissue damage. To reduce this collateral damage, a method for isolated skin irradiation was introduced. Prior research applied high single doses of radiation (≤80Gy) to the skin in order to study wound healing following radiation-induced dermatitis [2]. In an effort to further isolate the skin, the mouse is anesthetized, and the dorsal skin is stretched and secured using either clamps or sutures [1]. This allows targeted radiation of the stretched skin, minimizing damage to adjacent organs. This technique also enables uniform irradiation of the entire dorsal skin, allowing for multiple technical replicates from a single mouse, thereby reducing the number of animals required for statistically significant experimental outcomes.

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Abstract 22 (Federspiel *et al.*)

Postmortem retrograde-dye perfusion of the human heart – First steps in body donors towards a postmortem functional assessment of the heart.

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The death statistics can help to guide different research fields. For example, it allows us to identify for which disease assessment of new therapeutic options is mostly needed. [1] Hence, it is essential to determine the cause of death as accurately as possible, e.g. differentiating between death due to coronary heart disease and death due to heart valve disease instead of just diagnosing a 'cardiac death'. Despite the autopsy being considered the 'gold standard' [2] to determine the cause of death, there are conditions in which subsequent analyses are necessary. To diagnose a so called 'sudden cardiac death', for example, toxicological analyses are recommended by current guidelines [3]. Besides intoxications, a variety of conditions is hardly associated with gross findings. One example would be aortic regurgitation due to discrete geometric changes of the aortic root, like dilatation of the sino-tubular junction [4]. So, despite subsequent analyses, diagnostic gaps during necropsy remain, especially regarding functional pathologies and pathophysiologies of the heart valve apparatus due to lack of circulation. To diminish this 'functional' gap a first pilot study in human body donors was conducted [5]. The retrograde dye perfusion of the ascending aorta was found to provide hints regarding aortic valve coaptability [5]. In a follow-up study, the feasibility of the aortic root and left ventricular pressurization using a roller pump system was assessed. The present poster summarizes how the circuit was installed and which analyses can be done. By that, this study demonstrates how human body donors can be used to get insights into the functional anatomy of the heart valve apparatus. The need for research in humans is underpinned by striking anatomical differences between human and pig hearts [6] although the porcine hearts are well-established in the training of cardiac surgeons [7]. In summary, further research focusing on assessing the human functional anatomy is required to further improve our understanding of functional aortic valve disease.

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