Part I – Short Summary

1. Original Objectives and Scientific/Technical Background

1.1 Scientific and Technical Background

The project was motivated by the lack of suitable in vitro models to investigate the pathogenesis of non-alcoholic steatohepatitis (NASH) and inflammatory bowel disease (IBD), as well as to test relevant therapeutic compounds. The main challenges were replicating the complex tissue architecture and the disease-relevant immune responses. Through the development of organ-on-a-chip (OoC) technologies and the integration of appropriate cells, the project aimed to create an innovative platform to address these challenges.

1.2 Objective

The "Immune Avatar" project aimed to develop an in vitro drug-testing platform based on a microfluidic multi-organ chip. This platform was designed to replicate the interactions between liver, adipose tissue, and gut, particularly focusing on immunological and inflammatory processes related to NASH and IBD. The goal was to create a human-relevant test platform for personalized testing of different therapeutic options, with the potential to reduce animal experimentation. In the sub-project led by the University Hospital Jena (UKJ), the focus was on developing models for both NASH and IBD.

1.3 Results

1.3.1 WAT-on-Chip model by integrating immune cells

Adipose tissue plays a key role in immune-metabolic homeostasis, and insulin resistance is closely linked to the phenotype of the stromal vascular fraction (SVF) within the tissue. Using our previous WAT-chip that contained only human primary adipocytes, we developed an autologous and immunocompetent adipose tissue. Using a PDMS chip, adipocytes and SVF were loaded into a hyaluronic acid and collagen-based hydrogel, allowing for controlled concentrations and avoiding high pressures during seeding. This setup enables the evaluation of cell types in disease models or drug testing, as well as immune cell perfusion. The combination of SVF and an endothelial barrier significantly enhanced adiponectin secretion, particularly when tissue-resident macrophages were included. The phenotyping of macrophages and quantification of crown-like structures, along with cytokine profiling, offer insights into the potential mechanisms triggering insulin resistance. Lastly, we performed the perfusion of T-cells, isolated from the blood of the same patient, and we were able to detect T-cell migration from the vasculature-like perfusion to the tissue compartment [1].

1.3.2 Development of a multi-organ platform

The primary challenge in developing multi-organ chip platforms was determining the optimal cell culture media to maintain the function of various cell types. In this project, the focus was on connecting liver and adipose tissue models on a chip while ensuring that both tissues remain functional. The key cell types involved include white adipocytes, microvascular endothelial cells, and HepaRG cells (a liver cell line). Different combinations of adipose (WAT), endothelial, and liver-specific media were tested to assess their impact on cell viability, marker expression, and morphology.

In the first stage of the project, adipocytes and hepatocytes were vascularized separately and connected using a modular "Lego-plug and play" approach. Before optimizing the connection between the two organ models, it was crucial to evaluate how the secreted products from white adipose tissue (WAT), known as the conditioned medium, affect the liver chip. This conditioned medium was produced from adipose tissue samples obtained from two donors, both with and without the presence of endothelial cells. Several key parameters were measured, including glycerol secretion, glucose consumption, lactate production (indicating metabolic rate), and the secretion of DPPIV by endothelial cells. The presence of this enzyme was also analysed on the membrane of primary human adipocytes. These data were compared with previous research, noting that insulin and glucose levels were adjusted in the media.
Adipocytes and endothelial cells in the WAT-chip were nourished using media designed for both cell types. WAT media, supplemented with endothelial cell-specific nutrients, proved to be the most effective in promoting the viability of both cell types. This was also reflected in enhanced glycerol secretion, improved morphology, and more efficient fatty acid uptake and release. Meanwhile, HepaRG cells cultured on the liver chip, with a collagen-coated membrane, exhibited similar morphology across different media types (WAT media, endothelial cell media, and liver-specific media), maintaining functionality for up to five days in culture. This was achieved by perfusing the liver compartment with liver-specific media, while the WAT or endothelial media was used in the media channel.
The development of the connection workflow involved integrating two organ models (liver and adipose) within a single chip or in a modular multi-organ system. Both chips were produced using PMMA, making them less prone to chemical absorption. Herein, the design took the organ scaling, oxygen availability and possible perfusion strategies, linear and recirculation, into consideration. Both perfusion strategies yielded a viable liver and adipose tissues, as confirmed by calcein staining and immunofluorescence staining of albumin, e-cadherin, CD31, after 5 days of 2-organ connection.
In summary, this project established a functional and stable liver-adipose physiological model, highlighting the importance of media composition and modular design in multi-organ chip platforms.

1.3.3 Testing personalized therapeutic approaches for chronic inflammatory conditions

We characterized the adipocytes' viability, morphology, and functionality on-chip at different time points and their response to ß-adrenergic- as well as pro-inflammatory stimulation (LPS and TNFa). To assess the impact of donor-variability, we also compared how cells from different donors perform in the same experimental set-up [1]. Due to time constrains, this was not performed on the multi-organ platform.

1.4 Conclusion

The EKTU subproject contributed to developing a multi-organ chip platform by modelling liver and adipose tissues and its vascularized connection. The project focused on integrating white adipocytes, endothelial cells, and HepaRG liver cells into a functional system using a modular approach. It optimized media composition for both tissues, enabling stable perfusion and cell viability. This model provides a foundation for testing personalized therapies, particularly for NASH and IBD disease studies, and reducing animal testing in preclinical research.

Rogal, Julia, Julia Roosz, Claudia Teufel, Madalena Cipriano, Raylin Xu, Wiebke Eisler, Martin Weiss, Katja Schenke-Layland, and Peter Loskill. 'Autologous Human Immunocompetent White Adipose Tissue-on-Chip'. *Advanced Science* 9, no. 18 (2022): 2104451. https://doi.org/10.1002/advs.202104451.

Schlussbericht zum Verbundvorhaben Immune Avatar

Projekt:
"In-vitro-Challenge Projektphase - Entwicklung einer in-vitro Wirkstoff-Testplattform zur patientenspezifischen Modellierung der nichtalkoholischen Steatohepatitis und chronischer Darmerkrankungen in einem immunkompetenten Leber-Fett-Darm Organmodell"

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1. TASK PRESENTATION, REQUIREMENTS, AND PROJECT PROGRESS

1.1 Tasks

The "Immune Avatar" project aimed to develop in vitro models for investigating the fundamental mechanisms of non-alcoholic steatohepatitis (NASH) and inflammatory bowel disease (IBD) to improve understanding of these diseases and test new therapeutic approaches within these model systems. Despite significant progress in diagnosing and understanding these chronic conditions, current technologies are inadequate for realistically reproducing human immune diseases in vitro. This gap arises from biotechnological challenges due to the complexity of tissue organization, diverse biological immune responses, and high interindividual variability.

During the preceding exploratory phase, key prototypes were developed, including a human autologous white adipose tissue model and a vascularized 2-organ platform, connecting the white adipose-liver liver models. A central challenge was optimizing these models to reliably replicate the versatility and complexity of human immune responses, providing a foundation for developing and testing new therapies. Connecting these models will allow the investigation of interactions between liver and adipose tissues under pathophysiological conditions.

To address this gap, the project seeks to develop human-relevant technologies that can realistically replicate immune-mediated processes in metabolic diseases. A patient and disease-specific model for white adipose tissue will be established to study human cell and tissue responses to pathogenic and inflammatory stimuli, enabling the testing of novel therapeutic interventions. The project will leverage innovative organ-on-chip technologies to gain new insights into the pathogenesis of metabolic diseases, accelerating the development of more effective therapies. Particularly the development, use and the plan for technology transfer of a multi-organ platform is foreseen.

1.2 Conditions for conducting the proposed work

At the Eberhard Karls University of Tübingen, a microphysiological platform was developed to integrate adult human white adipose tissue. The WAT-on-a-Chip system features tissue-specific chambers connected by microchannels resembling blood vessels and protected by a semipermeable membrane. Using specialized protocols, functional 3D adipose tissue can be created and maintained for over a month. This platform enables the monitoring of key physiological aspects, such as fatty acid metabolism.

This technology was extended to create a multi-organ chip platform, combining Tübingen's WAT model with Jena University Hospital's liver model. The scaling was adjusted to mimic in vivo conditions, with a flexible connection between liver and adipose compartments. Both liver cells and functional adipocytes were successfully cultivated, enabling studies of the liver-adipose tissue interaction under physiologically relevant conditions.

1.3 Scientific and technical background

The molecular mechanisms of inter-tissue communication are essential for understanding the insulin resistance triggering events. With its multi-organ-chips, the

organ-on-a-chip technology provides promising in vitro models for studying the specific inter-tissue crosstalk avoiding the inaccessibility and non-human nature of animal models, and the limited sampling possibilities and inter-individual variability of clinical studies. There are several models for designing microphysiological systems according to the specific study needs. The general approaches for the integration to multi-organ devices are the (i) static systems, where multiple tissues are integrated into a single device connected to each other, (ii) semi-static systems, where tissues are interconnected via a fluidic network with Transwell®-based tissue inserts; and (iii) flexible systems where individual organ/tissue specific platforms are joined together using flexible micro-connectors.

While liver and white adipose tissue (WAT) models have been developed and are available for research, a critical gap existed in creating immune-competent versions of these models. Traditional liver and WAT models lack the incorporation of immune components, which are essential for studying the complex immune responses involved in diseases like non-alcoholic steatohepatitis (NASH) and other metabolic or inflammatory conditions. These diseases heavily involve immune and inflammatory pathways, and without immune cells integrated into the models, key aspects of their pathophysiology remain unaddressed.

Additionally, robust platforms that connect both liver and WAT in a physiologically relevant way are also missing. The interaction between these two organs plays a crucial role in metabolic and inflammatory diseases, where crosstalk between liver and adipose tissue, mediated by immune cells, is critical for disease progression. A multi-organ system that links immune-competent liver and WAT models would provide a more comprehensive and realistic platform for studying such interactions, advancing both basic research and therapeutic testing.

1.3.1 White adipose tissue models

Developing microscale platforms that mimic the physiological environment of white adipose tissue (WAT) is essential for long-term culture and disease modelling, particularly for conditions like obesity and diabetes as well as NASH and IBD. By leveraging advanced techniques in microfabrication, biomaterials, and tissue engineering, organ-on-a-chip (OoC) technology has emerged as a promising tool for mechanistic studies and pharmaceutical testing. This approach enables the creation of perfused microenvironments that replicate in vivo-like tissue structures, offering valuable insights into multi-factorial diseases and personalized medical treatments.

Despite significant advancements in OoC research for various organs and tissues, WAT has been relatively neglected, with only a few platforms addressing its complexities. Previous efforts involved injecting pre-adipocytes or primary adipocytes into microfluidic chambers, but these did not replicate the in vivo physiology of WAT. Recently, a novel approach by Harms et al. used multiwell plates for culturing mature adipocytes, yet these models still lacked key physiological features.

Our team published the first OoC platform that integrates human primary mature adipocytes into a perfused microfluidic chip. The platform features multiple tissue-specific chambers connected to a vasculature-like microchannel, which shields the cells from shear stress. Using optimized isolation and injection protocols, 3D microtissues are formed in individual chambers, enabling the long-term viability and functionality of adipocytes for over a month. The platform also facilitates real-time monitoring of cellular functions, such as fatty acid metabolism and drug responses, by analysing media

effluents and utilising the optical accessibility of the tissue chambers. This innovation offers a powerful tool for disease modelling, drug testing, and personalized medicine.

1.3.2 Multi-organ models for metabolism studies

During the "exploratory phase," a prototype was developed that combines the liver model in the biochip format from the University Hospital Jena with the adipose tissue model from the Eberhard Karls University of Tübingen. The scaling was carefully chosen to accurately replicate in vivo conditions. The size of the cavity for cultivating the liver model was adjusted and reduced to accommodate the use of primary donor cell material. A flexible approach was used for connecting the two organ compartments, utilizing different types of connectors. This allows both tissues to be established independently at first. In the second step, successful cultivation of liver cells and functional adipocytes was achieved in their respective compartments within the two-organ chip.

1.4 Project Planning and Progress

In the ImmunAvatar project, EKUT focused on developing an autologous, vascularized, and immunocompetent white adipose tissue chip and integrating it with a liver chip, building on the liver chip development at Universitätsklinikum Jena (UKJ) to establish a two-organ platform. Additionally, we designed an immune cell reservoir to enable stable immune cell perfusion over extended periods. Our contributions were directed towards work packages 2, 5, and 6. This report outlines our organizational strategies to achieve the objectives of each work package, detailing the goals and methodologies of each research phase.

In the context of the project ImmunAvatar, EKUT focused on the development of an autologous, vaslcularised and immunocompetent white adipose tissue chip, on its connection to the liver chip based on the liver chip development in Universitätsklinikum Jena (UKJ) to build a 2 organ platform as well as on the conception of an immune cell reservoir that would allow stable immune cell perfusion for longer periods. Therefore, we contributed to work packages 2, 5 and 6. Herein, we describe how we organized the work to meet the objectives of each work package.

WP 2 - Development of WAT Model from Primary Donor Cells (EKUT)
Objectives and plans

The WAT-on-Chip prototype previously developed at EKUT was optimized and further advanced by integrating tissue resident immune cells, endothelial cells and circulating immune cells. To achieve this, adult human adipocytes and microvascular endothelial cells (MVECs) were isolated from biopsies using established protocols. Protocols for PBMCS and monocyte isolation were implemented due to the contribution of UKJ. The adipocytes were then embedded in a hydrogel and injected into the WAT chips, with MVECs lining the vessel-like channels. Initial logistics for biopsy transportation were established to allow building an autologous model and obtain adipose tissue, skin and blood from the same patient. This was particularly challenging during the pandemic times, particularly because our biopsies are obtained from non-urgent surgeries. Adipocytes were co-cultured with the stromal vascular fraction of the adipose tissue or

tissue-resident macrophages isolated from it. The SVF and macrophages were injected into the tissue chamber in a differentiated state at a physiological ratio with adipocytes. Alternatively, we introduced undifferentiated (monocytes) or in early differentiation stages through the blood vessel channels, based on processes established at UKJ. Additionally, techniques for circulating PBMCs through the vessel channels, previously established in similar OoC platforms at UKJ and EKUT, were adapted and optimized for the WAT-on-Chip model.

Results and Assessment

By developing the logistics of obtaining different cell types from the same donor, we would achieve a mix-and-match approach for the integration of the endothelial barrier, immune cell perfusion, adipose tissue macrophages, blood-derived monocytes differentiated to macrophages as well as T-cells.

Using positive controls of inflammation (TNFa and LPS) and specific cell types, we would demonstrate the possibility of building healthy and diseased WAT phenotypes, that could then be coupled to the liver and intestine models.

WP5 - Establishment of a Multi-Organ Platform (EKUT/UKJ)

Objectives and plans

Prior work using a flexible plug-and-play approach for connecting individual organ platforms was to be further developed and optimized to create a 2-organ platform. Due to time constrains and challenges in technology transfer, we focused solely on the 2-organ connection and focused on 2 different approaches: modular approach and inbuilt approach. The modular approach allowed separate assembly and maturation of organ platforms before combining them. Connection elements with minimal dead volume were further developed, with special attention given to their design and optimization to perfuse circulating immune cells without compromising their vitality and functionality. To minimize the absorption of hydrophobic molecules (e.g., cytokines, fatty acids, hormones), PDMS was avoided in favour of non-elastomeric materials (PMMA). The developed biochips were used for stable cultivation of fat-liver systems, with adjustments made to the nutrient medium composition and optimization for co-cultivation. Based on established protocols for individual systems already in use at UKJ and EKUT, perfusion processes and microchannel architectures were established and optimized for the injection and circulation of PBMCs. As a risk management task, we developed in parallel the in-built approach where both organs are in the same chip. This provides less flexibility and it is more challenging in terms of cell source logistics, but it allows a more simple handling, lower training times for new uses and smoother technology transfer.

Results and Assessment

The expected results are (i) a developed a cassette and connection module for 2-organ connection that allows vascularization of each tissue for 5 days; (ii) an inbuilt 2 organ-chip; and (iii) and immune cell reservoir that allows stable and viable immune cell perfusion.

WP 6 - Testing of Individualized Therapeutic Approaches (UKJ/EKUT/ITEM/Charité)

Objectives and plans
To evaluate the potential of our models to be used for testing individualized therapeutic approaches, we selected drugs, pro-inflammatory and anti-inflammatory stimuli. Upon such conditions are able to evaluate the model responses on the white adipose tissue, particularly on the response to insulin, cytokine release and crown-like structure formation.

Results and Assessment
The in vitro test platform should be evaluated for drug testing for the generation of metabolic disease phenotype.

1.5 Collaboration with other institutions

We, **EKUT (Eberhard Karls University of Tübingen)** focused on the optimization and further development of a fat tissue-on-chip model and a multi-organ platform. This work involved implementing flexible plug-and-play connection elements to enable the successful integration of separate organ models, as well as developing immune cell perfusion processes and adjusting nutrient media for co-culturing various organ models. EKUT also contributed to the technical optimization of the platform to ensure stable and functional cultivation of the organ models. The follow-up studies are underway and characterized, with ongoing work on the transfer phase. This phase is highlighting optimization potential and the need for extensive training, as originally envisioned. Current efforts are addressing challenges related to scaling, transferability, and pumping strategies.

UKJ (Universitätsklinikum Jena)
UKJ was crucial in the development and implementation of the liver-on-chip and intestine-on-chip models. Their tasks included the cultivation and optimization of the individual organ models, as well as integrating these models into a multi-organ platform. UKJ contributed to the development of the new biochip with two membranes and the creation of a central vascular area. Additionally, UKJ was responsible for testing therapeutic options for treating chronic inflammatory liver and intestinal diseases (NASH and IBD) using human organ-on-chip models.

Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM)
ITEM focused on modelling inflammatory bowel diseases (IBD). Their tasks involved establishing immune-competent in-vitro intestinal models that replicate both acute and chronic inflammatory responses. ITEM was responsible for isolating and providing PBMCs and other immune cells and developed protocols for co-culturing intestinal cells with lymphocytes to better understand disease mechanisms and test potential therapeutic approaches.

Charité – Universitätsmedizin Berlin
Charité was primarily responsible for developing a murine liver-on-chip model and handling the clinical aspects of the project. Charité supported the testing of therapeutic

approaches by providing information on disease progression, comorbidities, and other clinically relevant data.

Collaboration of Partners

The collaboration among the partners was conducted with close coordination and a well-organized division of labour. Regular meetings and workshops facilitated the exchange of progress and results and allowed for adjustments to work plans. The combination of the partners' diverse expertise led to significant advancements in the development and testing of new therapeutic approaches. UKJ focused on model development and testing, ITEM on immune cells and intestinal models in the Transwell format, EKUT on developing a fat tissue model and technical optimization of the multi-organ platform, and Charité on developing murine liver models and comparing them with the human liver-on-chip model. This synergistic collaboration was crucial for the project's success.

Project meetings

To coordinate the work, joint project meetings were held on:

- August 13, 2020
- December 10, 2020
- September 15, 2021
- May 10, 2022
- May 23, 2023
- September 14, 2023

2. PRESENTATION OF PROJECT RESULTS

2.1 Project Results

EKUT made significant progress in developing human-relevant in-vitro models that not only contribute to the reduction of animal testing but also enable more precise testing and development of new therapeutic approaches.

The following sections provide an overview of the results related to white adipose tissue, the multi-organ platform, and immune cell perfusion. This presentation highlights the scientific and technological advancements achieved during the project and offers insights into the methods employed and the outcomes attained.

2.1.1 White adipose tissue model: autologous and immunocompetent

The results of this WP are fully published in Rogal et al. 2022[1]. Briefly, the PDMS chip has a design that allows a sequential loading of high density of adipocytes and SVF in a controlled concentration and within a hydrogel (Hyaluronic-acid and collagen based), while avoiding exposing them to high pressures during seeding (**Figure 1A**). As such, it is possible to evaluate the role of each cell type of combinations of them in a particular disease or pharmacological assessment (**Figure 1B**) or to monitor immune cell perfusion (**Figure 1C**). We observed that the combination of SVF and an endothelial barrier had a higher impact on adiponectin secretion than each of the components separately (**Figure 1D**). Particularly, the inclusion of tissue resident macrophages, its phenotyping and the relative quantification of crown-like structures formation (**Figure 1E**) and its respective cytokine profile (**Figure 1F**) paves the way to evaluate possible triggering mechanisms of insulin resistance.

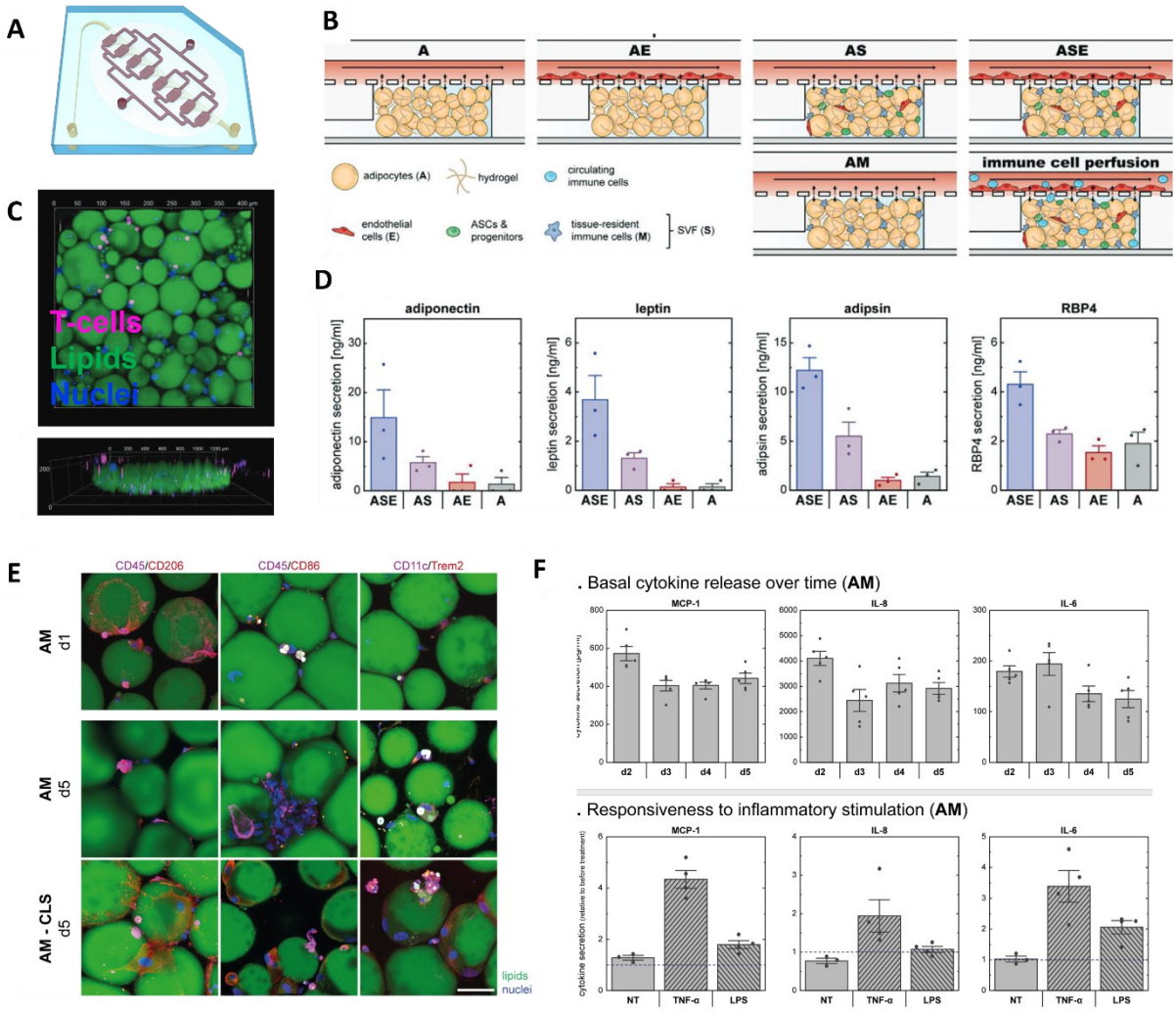


Figure 1. Overview of the mix and match approach for the human autologous immunocompetent white-adipose tissue chip. Scale bars = 50 µm

2.1.2 Establishment of a Multi-Organ Platform

The major challenge of multi-organ chip platforms is the definition of the optimal cell culture media that assures the function of all cultured cell types. For that, we accessed the viability, marker expression and morphology of white adipocytes, microvascular endothelial cells and HepaRG cells on chip and the culture with its media or WAT media, endothelial cell media and mixtures thereof. Functional adipocytes and hepatocytes were separately vascularized and connected using a Lego-plug and play approach.

Adipose- and liver-chip connection: establishment of the liver-adipose physiological model

Before the connection strategies are optimized and implemented, it is of major interest to evaluate the effect of the conditioned medium, produced by the white adipose tissue, that could be used separately for testing its effects on the liver chip. A fully characterized sterile secretome from tissue from 2 donors, with and without the presence of endothelial cells (**Figure 2A and B**) was produced. The tissue was also evaluated on glycerol secretion (**Figure 2C)**, glucose consumption and lactate production (metabolic rate, **Figure 2D**), DPPIV secretion by endothelial cells and presence at the membrane of the human

primary adipocytes (**Figure 2E**). This data complements the work from the manuscript Rogal et al. 2022 because the media was adjusted to 60 pM Insulin, instead of 60 nM and glucose levels reduced to 11 mM

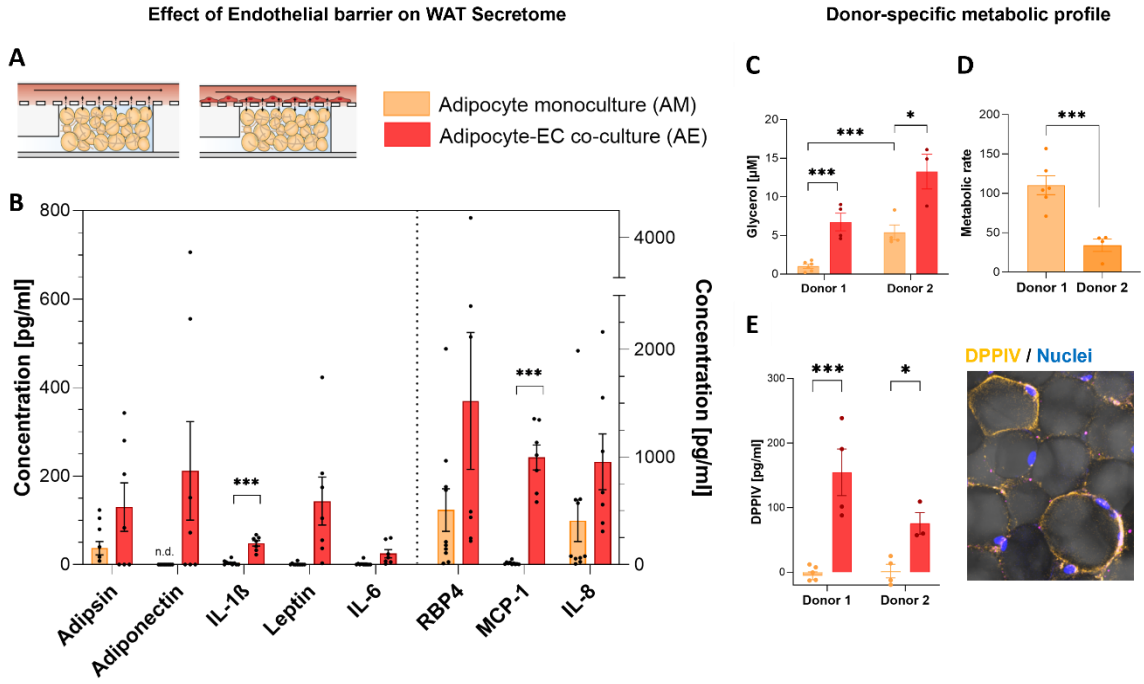
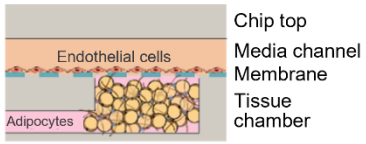
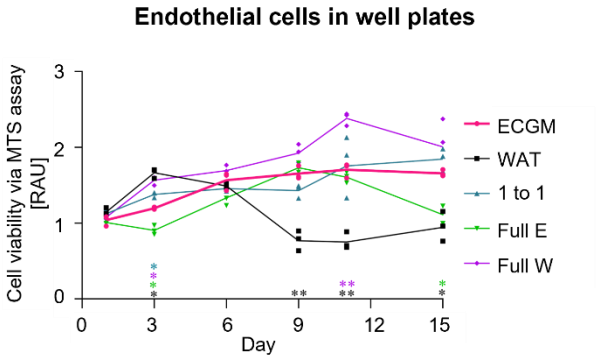


Figure 2. Effect of including endothelial cells in the WAT-chip secretome and the impact of interdonor variability for basal lypolyis and DPPIV secretion by endothelial cells and expression by adipocytes.

In the WAT-chip, adipocytes and endothelial cells were both nourished from the media channel of the WAT-chip. The WAT media containing endothelial cell supplements revealed to be the most advantageous regarding the viability of both adipocytes and endothelial cells (**Figure 3**) and also regarding glycerol secretion, morphology and fatty acid uptake and release (**Figure 4**). HepaRG cell line, cultured on the liver chip on a collagen coated membrane demonstrated the same morphology in both WAT-media and Endothelial cell media, similar to its culture in Williams E, up to 5 days in culture (**Figure 5**). This was performed by having liver media in the hepatocyte compartment and perfused with the WAT or endothelial media on the media channel.

ECGM	Commercially available endothelial cell media
WAT	Chip culture maintenance media as published by Rogal et al. (Rogal et al., 2020)
1 to 1	1:1 mixture of ECGM and WAT media (Rogal et al., 2022)
Full E	ECGM media containing all the supplements of WAT medium
Full W	WAT medium containing all the supplements of ECGM medium





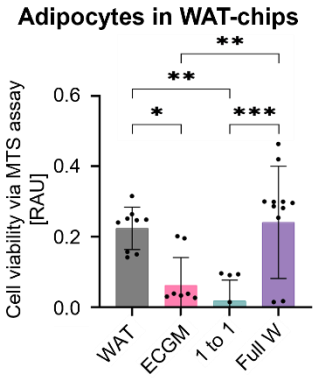


Figure 3. Media optimization and its effects on endothelial cells and adipocytes viability

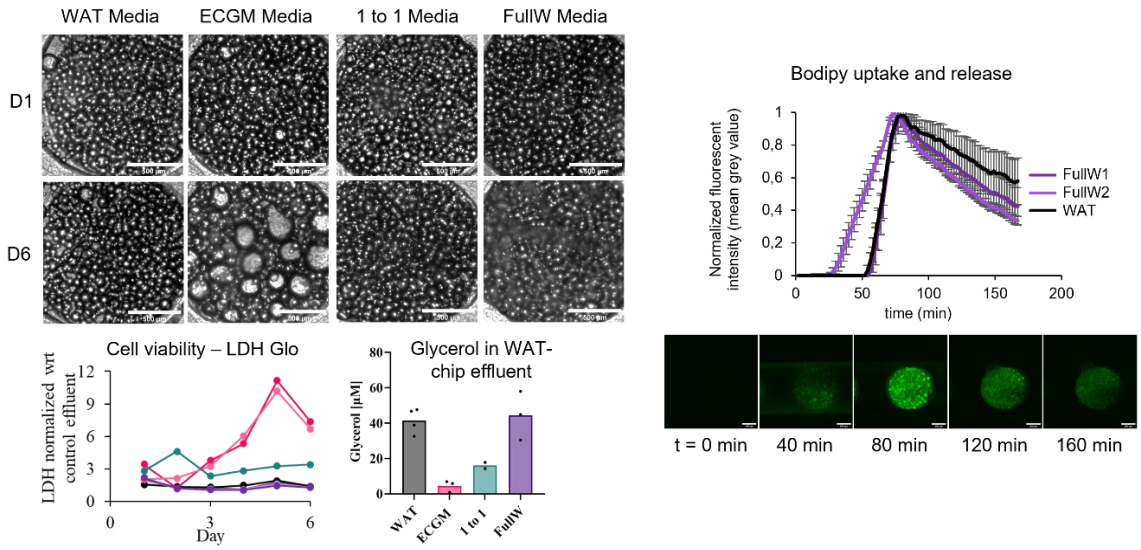


Figure 4. Effect of endothelial, liver and white adipocyte media on white adipocytes morphology, viability, basal lipolysis and fatty acid uptake and release kinetics.

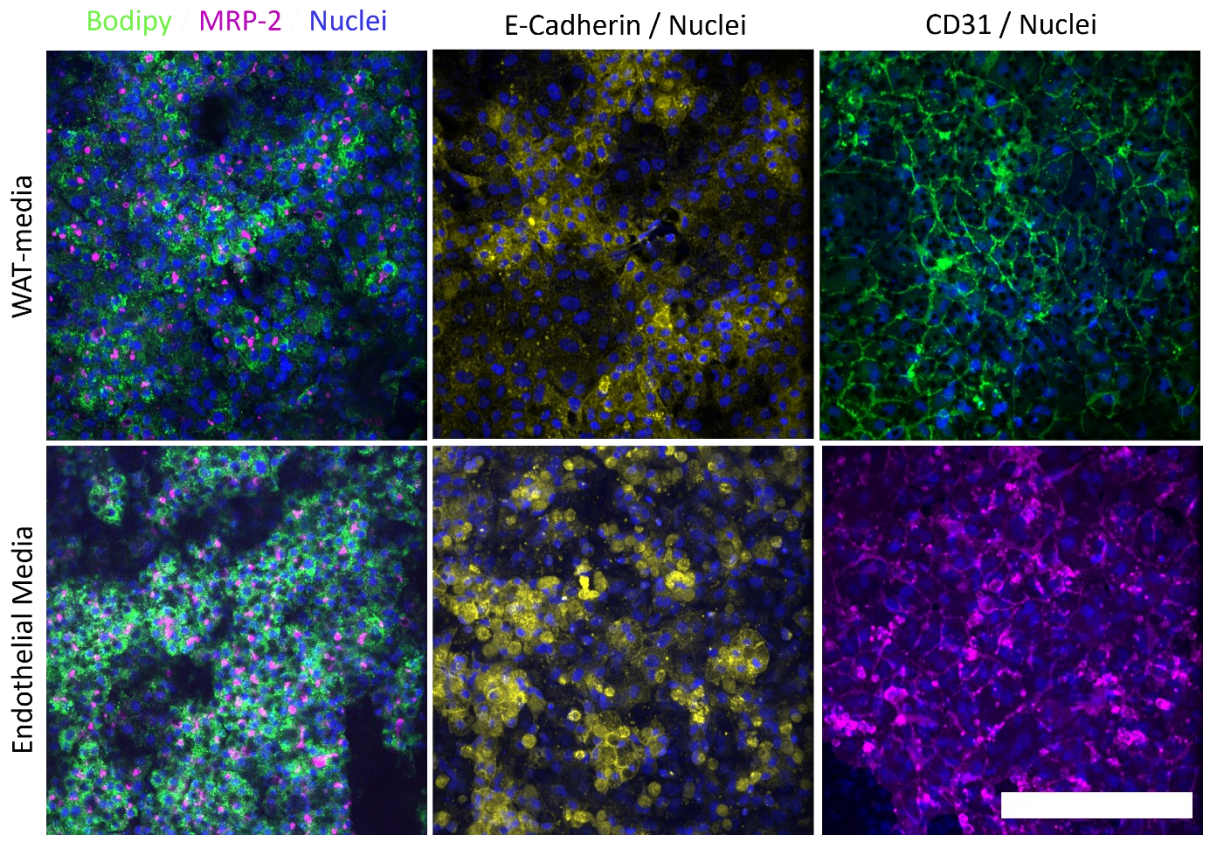


Figure 5. Evaluation of cell specific markers in chips cultured separately with WAT media and endothelial media

The development of the connection workflow covered the system used in WP1, with 2 organs in the same chip. Sensor integration and the challenges of assuring microfabrication protocols that do not compromise sensor performance, lead to the development of a modular multi-organ approach. Desing considerations, along with organ scaling, allowed for stable liner perfusion and recirculation. **Figure 6A** shows a schematic overview of the closing mechanism by applying pressure at the critical contact points and a side view scheme of the connection of 2 systems with the vascular perfusion. 3D printing allowed for quick, efficient and user-friendly connection and alignment of chips within the cassette. 3D printed features include 3D printed threads to reduce the number of parts to be connected, to allow the alignment and fixation of the connection channel and allow for optical non-invasive sensor integration and a closed connected chip using even pressure by combining screws and springs and integration of a reservoir holder at a defined distance (**Figure 6B**). Recirculation perfusion setup using a fixed 500 µL reservoir that allows stable perfusion over 7 days in culture assuring both white adipose tissue and liver tissue viability, accessed by Calcein (**Figure 6C**).

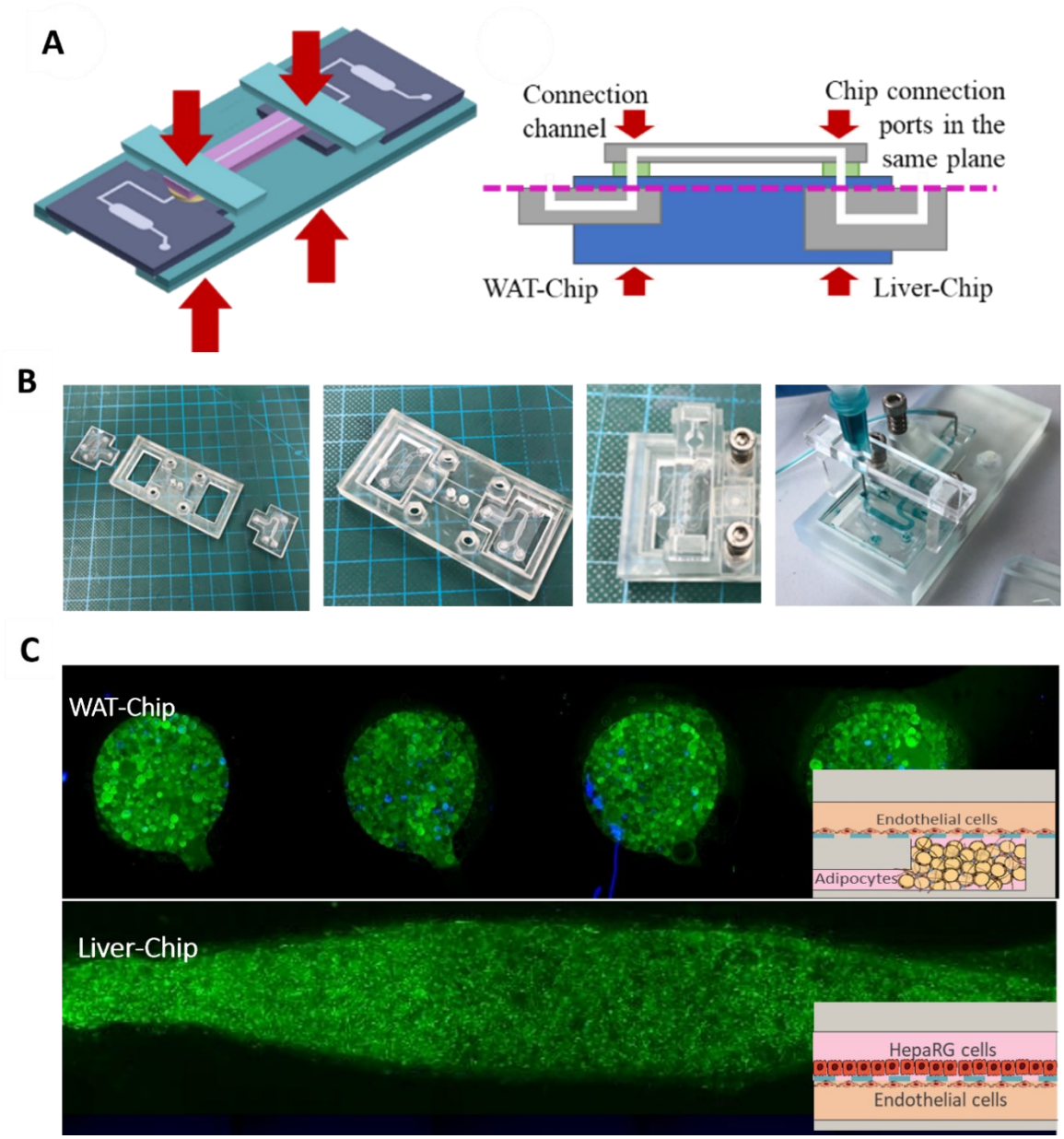


Figure 6. Modular approach to the multi-organ platform demonstrates white adipocytes and HepaRG cells viability after 5 days in connected culture.

Figure 6 demonstrates the presence of a stable endothelium, cultured on fibronectin coated PET and PC membranes in the liver and white adipose tissue respectively. On the top and left, the endothelial barrier on top of the adipocytes shows a stable CD31 and VE-Cadherin expression while the adipocytes retain its morphology and unilocularity. On the right left we can observe the same on the liver chip, covering both the HepaRG cells or the connecting channels.

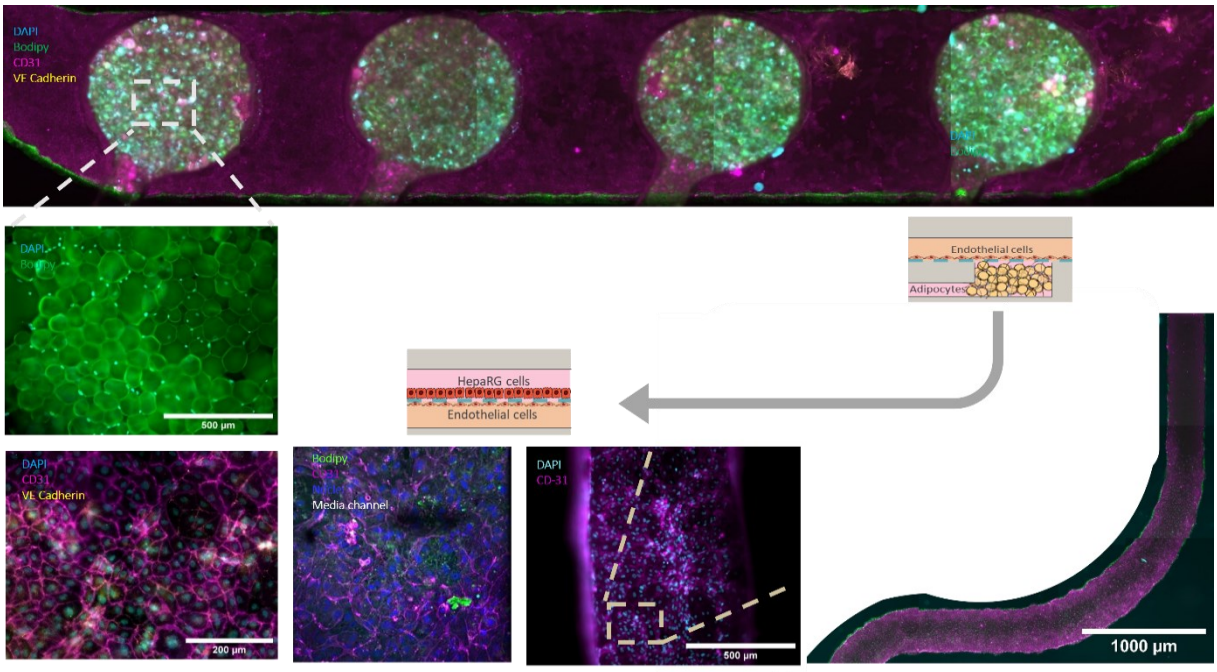


Figure 7. Expression of cell specific markers after 5 days of endothelized WAT-Liver culture

Establishment of the liver-adipose physiological and physio-pathological

The functional demonstration of the 2-organ connection was achieved in a fully reendothelized white adipose tissue and liver tissue. The liver was connected downstream of the white adipose tissue under linear perfusion, yielding a higher accumulation of intracellular fatty acids in the connected hepatic tissue (**Figure 8A**). The lipid droplets can be observed under laser scanning microscopy (**Figure 8B**) and quantified using widefield microscopy (**Figure 8C**). This corroborates the observation that triglyceride secretion is increased in the WAT-Liver connected set-up compared to the liver alone, which is observed both after 24h of culture and after 6 days of connected culture (**Figure 8D**).

Although we obtained results from the connection platform, the yield of successful connections was relatively low (< 50%), and the protocols were too complex for transfer to our partners in Jena and Berlin. The major challenge is a sterile and bubble free connection. If the connection is not bubble free, the endothelial layer will be lost and consequently affect the viability and quality of the whole system. This would have required intensive training, which was not initially planned. As a result, we also focused on developing our in-house 2-organ system. However, due to time constraints, we were unable to achieve the level of technology transfer we had aimed for.

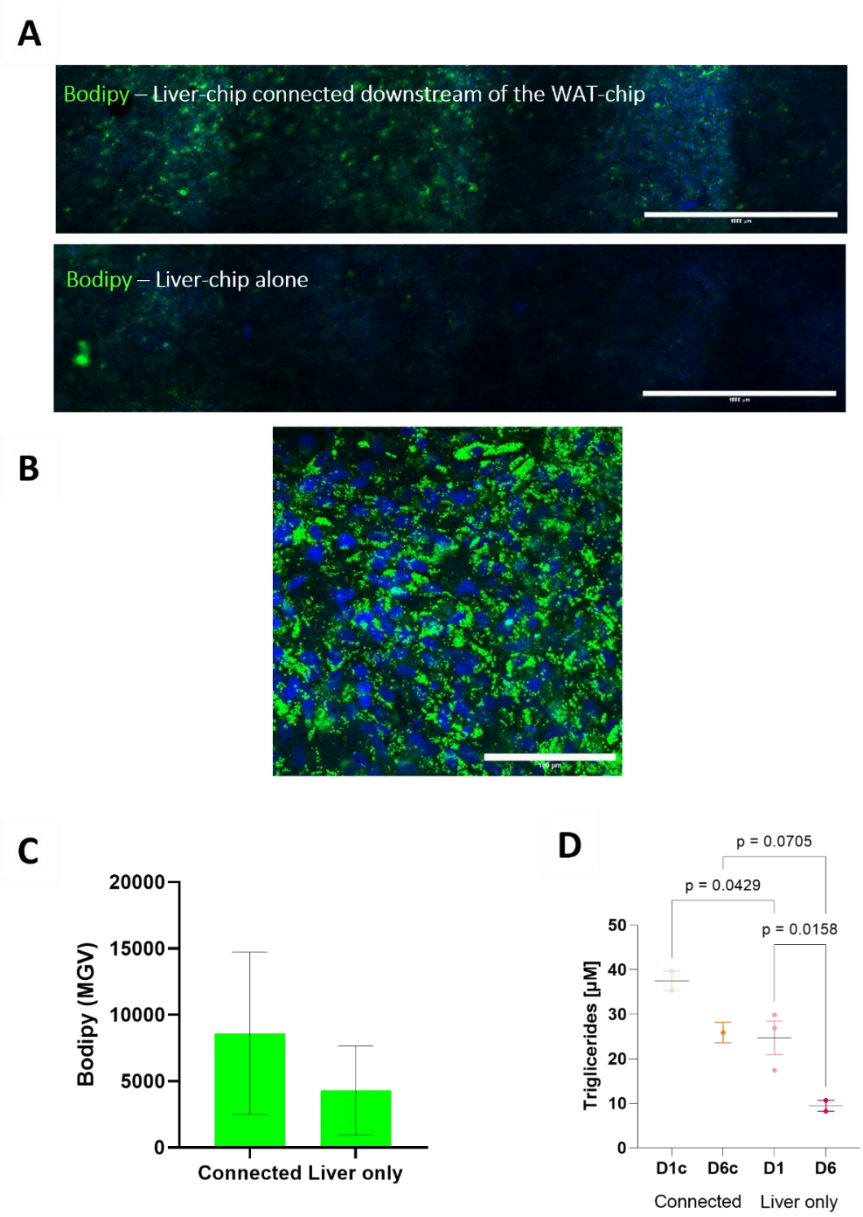


Figure 8. Effect of the WAT on the liver-chip after 5 days of endothelized connected culture demonstrating and increase on intracellular lipid content and basal triglyceride release.

2.1.4 Development of a robust immune cell perfusion strategy

Integrating circulating immune cells into organ-on-chip (OoC) models presents significant challenges due to the fragile nature of these cells, their tendency to attach to irregular surfaces, and their proclivity to settle in low-flow-rate areas. Furthermore, most platforms are not designed to support the long-term suspension and perfusion needed for these cells, particularly peripheral blood mononuclear cells (PBMCs). To address these issues, an immune cell holder was developed to integrate immune cells into a multi-organ chip (MOC) platform, facilitating long-term suspension and perfusion. This immune cell holder was designed with several key considerations and underwent multiple stages of conceptualization, simulation, and fabrication to optimize its function within the MOC system. Below, we provide an overview of the development process.

Concept of the Immune Cell Holder

The immune cell holder was conceptualized with the following goals in mind:

- Minimize sharp edges to prevent damage to immune cells.
- Protect immune cells from direct shear stress caused by fluid flow.
- Enable easy integration with various MOC platforms through flexible and user-friendly interfaces.
- Avoid the use of glass, given its limitations in biocompatibility and breakage risks.
- Ensure that there is sufficient upward flow to keep immune cells in suspension and prevent sedimentation.
- Design with a compact footprint of 25 by 25 cm, ensuring compatibility with standard MOC platforms.

The final design of the immune cell holder includes a cylindrical cell suspension chamber (CSC), where cells are confined between isoporous membranes. This chamber is housed within an outer mixing chamber that maintains a flow speed sufficient to counter the natural settling velocity of the cells, ensuring their continued suspension.

Flow Simulations for Design Optimization

COMSOL simulations were used to model the fluid dynamics within the immune cell holder, with a focus on achieving the necessary upward flow for cell suspension. The design initially comprised two compartments: a lower mixing chamber and an upper cell chamber, separated by a membrane. The simulations revealed that flow focusing within the CSC could be achieved with fluid velocities exceeding 15 µm/s, matching the settling velocity of immune cells.

By adjusting the gap between the CSC and the mixing chamber and extending the walls of the CSC into the mixing chamber, flow was further focused, ensuring cells remained suspended. These insights helped guide the development of the immune cell holder, and future iterations of the COMSOL model could introduce more complexity to simulate interactions between fluid and the mixer more accurately.

Fabrication of the Immune Cell Holder

The immune cell holder was fabricated as a cylindrical chamber with isoporous membranes on both sides to confine the cells while maintaining suspension. Two approaches to mixing and flow focusing were explored:

1. **Concentric Mixer Approach:** This design used an outer mixing chamber with a magnetic stirrer or motorized impeller to drive the fluid upwards through the CSC.
2. **External Pump Approach:** This alternative employed a miniature peristaltic pump to create upward flow across the membranes, without the need for an external mixing chamber.

Both designs were intended to interface with the MOC platform, allowing media to flow through the CSC and maintain cell suspension.

Concentric Mixer Approach

The concentric mixer approach evolved through several design iterations, each aimed at optimizing the mixing of the fluid and the stability of the system. The first iteration used a transwell-like insert to suspend the CSC in the mixing chamber, but this setup experienced issues with side access points, which led to leakage and dead volumes.

In later iterations, the access points were relocated to the top of the CSC, and the system was mounted onto the platform using metal cannulas. A magnetic stirrer was initially

employed to create upward flow, but this was later replaced by a motorized impeller, which provided stronger and more controlled flow. However, the introduction of membranes reduced the effectiveness of the upward flow, and the top-heavy design caused instability, requiring additional securing mechanisms.
Despite the improvements, the concentric mixer approach faced challenges in maintaining strong enough flow across the membranes and ensuring a stable, leak-proof connection to the platform.

External Pump Approach
The external pump approach used a miniature peristaltic pump to drive medium upwards through the CSC, eliminating the need for a separate mixing chamber. This design offered a simpler setup, with fewer components and less risk of instability. The use of a reservoir downstream of the pump also acted as a bubble trap, preventing microbubbles from activating the immune cells.
This approach was more straightforward and avoided many of the issues associated with the concentric mixer, such as dead volume and leakage. The external pump was controlled via a voltmeter, and early tests showed promising results in maintaining cell suspension without causing excessive shear stress.

System Characterization of the Immune Cell Holder
The immune cell holder was evaluated through a series of characterization tests to determine its performance in terms of fluid dynamics, particle suspension, and endotoxin levels. The results below provide insight into the holder's ability to maintain cell suspension, its comparison with commercial systems, and its cleanliness for biological applications.

Maximum Upward Velocity
To assess the fluid dynamics within the immune cell holder, videos were taken to measure the upward velocity of coloured water in the cell suspension chamber (CSC). These velocities were compared with the settling velocity of cells (V_t) in water, calculated using the following formula:

$$V_t = g * d^2 * \frac{(\rho_c - \rho_m)}{18 * \mu}$$

Where:

- g is gravitational acceleration,
- d is the cell diameter,
- ρ_c is the cell density,
- ρ_m is the medium density, and
- μ is the medium viscosity.

The mini-peristaltic pump produced an upward velocity of 2E-5 m/s, which was comparable to the settling velocity of cells. Other systems, such as the motorized impeller, achieved a higher velocity of 1.2E-4 m/s when set to maximum voltage. This tunability demonstrates the system's capacity to maintain immune cells in suspension, as the upward velocity can be adjusted to meet specific requirements.

Bead Flow Test
A bead flow test was conducted to validate the immune cell holder's ability to keep particles suspended. The test used a commercially available syringe pump (NeMix™,

Cetoni GmbH), which mixed a fluorescent bead solution. The expectation was that the effluent would have a uniform concentration of beads, reflected by consistent fluorescent intensity. However, the concentration of beads was not uniform, likely due to bead settling in the NeMix reservoir. This bead flow protocol can be adapted for the immune cell holder. In this approach, a high concentration of beads would be injected into the CSC at the start (t0), and phosphate-buffered saline (PBS) would be flushed through the micro-organic culture (MOC) system using a syringe pump. Regular effluent samples would be collected to monitor bead concentration over time. A decrease in bead concentration, aligned with the perfusion rate, would indicate that the beads remained in suspension and were progressively washed out of the system.

Baseline Endotoxin Measurement

Another key aspect of system characterization involved measuring endotoxin levels to ensure that the immune cell holder could be used safely in biological studies. The basic cleaning protocol for the immune cell holder involved wiping all parts with 70% ethanol, drying them in a biosafety cabinet, and then exposing the assembled holder to UV light for 30 minutes. After cleaning, the holder was filled with PBS, sealed with a rubber cap, and connected to pre-filled syringes. The setup was perfused at 37°C overnight, and effluent samples were collected the following day for endotoxin analysis. To further validate the cleaning process, the system was exposed to 1M NaOH for 1 hour, followed by two washes with endotoxin-free water. The system was then refilled with PBS and perfused again.

Endotoxin levels were measured in the effluent before and after NaOH treatment. The baseline endotoxin levels were observed to be at the lower limit of detection, at 0.17 EU/mL. This value is significantly lower than the recommended baseline endotoxin level of 2.75 EU/mL, which could potentially interfere with in vitro culture studies. The results confirmed that the cleaning process, which included ethanol and UV light treatment, was sufficient to achieve low endotoxin levels suitable for biological applications.

These tests confirmed that the immune cell holder system is capable of maintaining sufficient upward flow to keep immune cells suspended, while also being clean enough for use in biological applications.

Conclusion

Both the concentric mixer and external pump approaches provided viable methods for incorporating immune cells into the MOC platform. The concentric mixer offered better control overflow patterns but required more complex setup and adjustments, while the external pump approach was simpler, with fewer stability and fluid dynamic issues.

In the final approach, we integrated the immune cell reservoir into an expanded cassette module for space considerations (**Figure 9A**). We selected the concentric mixer approach (**Figure 9B**), where cells were shielded from shear forces and impellers by a membrane. For optimal mixing, the impeller was redesigned to generate upward flow while protecting the cells in the inner reservoir (**Figure 9C**). This design demonstrated homogeneous immune cell perfusion, maintained over 4 days at a concentration of 1 million cells/mL (**Figure 9D**). The integrated system featured an on-chip reservoir for circular perfusion, motor-driven impellers, safe suspension of cells, and in-line media changes or drug applications.

The immune cell holder represents a significant step toward enabling long-term immune cell suspension and perfusion within MOC platforms. Future efforts will focus on refining flow focusing and simplifying the system for easier technology transfer and broader application integration.

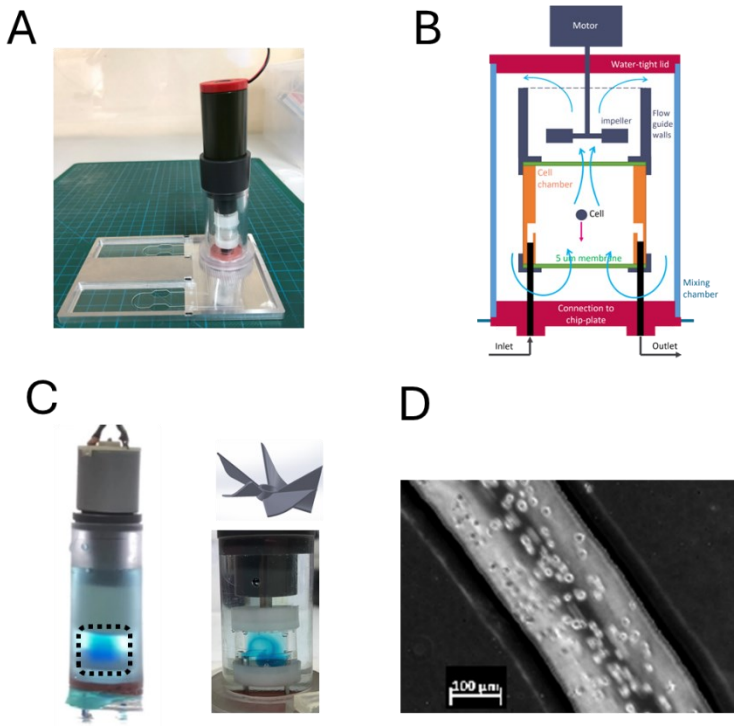


Figure 9. Immune cell reservoir concept and demonstration of the mixing principle

2.2 Key Items of the Financial Statement

For EKUT personnel funds amounting to 36 person-months (PM) at pay grade EG 13, totalling €225,254, were requested. Additionally, consumables totalling €68,257 were requested for project work. The consumables were used for microfabrication, molecular biology and cell biology research activities.

2.3 Anticipated Benefits and Usability of Results According to the Updated Exploitation Plan

The work carried out is both necessary and appropriate for contributing to the replacement and supplementation of animal testing. These in vitro models offer a humane alternative by providing realistic recreations of human organs. They enable detailed studies of disease mechanisms under controlled conditions, something that animal models often cannot achieve. Using human cells and replicating specific disease mechanisms creates an essential foundation for translating non-clinical research findings into clinical applications. This enhances the predictability of the effectiveness and safety of new therapies, contributing to more efficient and safer treatments.
The project allowed EKUT to develop and optimize the WAT-chip and the two-organ connection with the liver chip. The work from the autologous and immunocompetent

WAT-chip has been internationally recognized. Although we faced several challenges on the multi-organ platform, considerable resources were invested in its development. The immune cell reservoir, designed for stable mixing and maintenance of immune cells in suspension, required more resources than initially anticipated, which diverted focus from other tasks, such as Work Package 6. The COVID-19 pandemic and issues related to sourcing hepatic cells also presented significant constraints and we only used the HepaRG cell line in the 2-organ model.
Close collaboration between various research institutions and clinical partners, including the Charité in Berlin, facilitated the efficient and successful execution of these projects. Although we put significant efforts on the development of the multiorgan platform, it was not possible, during the course of the project to transfer it to Berlin and to Jena. Additional resources for training and technologies transfer should have been allocated. Therefore, EKUT tasks in WP6 were not performed. Despite the challenges posed by changes in operational techniques and the COVID-19 pandemic, the project was adapted flexibly, and most project goals were still achieved.

2.4 Expected Benefits and Usability of the Results in Line with the Updated Utilization Plan

2.4.1 Progress in the Field of the Project at Other Institutions

No results have been identified that would necessitate a modification of the project's implementation. The current state of science and technology is ensured through literature research in electronic databases such as PubMed. Additionally, participation in scientific events and conferences keeps the project up to date.

2.4.2 Publications of the results

This work has been presented internationally, particularly being selected for an oral presentation on the MPS Summit 2023 in Berlin and invited for a guest lecture at the Institute for Diabetes Research and Metabolic Diseases (IDM) from the Helmholtz Diabetes Center in the university of Tübingen and an invited talk at the 26th International Workshop on Diabetes and Related Metabolic Disorders at the University Miguel Hernandez in Elche, Spain.

The data was published in one peer-reviewed journal:
Rogal, J. et al. Autologous Human Immunocompetent White Adipose Tissue-on-Chip. *Advanced Science* **9**, 2104451 (2022).

Two additional manuscripts are currently in preparation. The first focuses on the 2-organ connection, examining Losartan as a therapeutic agent. In this study, Losartan is bioactivated by the liver and subsequently reduces lipolysis in adipose tissue. While most of the data for this manuscript has been analysed, the therapeutic proof-of-concept is still pending. This work aligns with the objectives of WP6, which has not yet been completed. The second manuscript will cover immune cell perfusion within a 2-organ platform.