Chapter I

General Introduction

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Microvascular diseases

In the last decades, great progress has been made in the treatment of the atherosclerosis-driven macrovascular diseases that are a leading cause of death worldwide. However, it is now well established that diseases of the microvasculature contribute to mortality in equal proportions.

In the cardiovascular field, heart failure (HF) is a major global health care problem with high morbidity and mortality, already affecting more than 23 million people worldwide [1]. Traditionally, most attention was paid to HF with reduced myocardial contractile function, left ventricular dilatation and reduced ejection blood volume (HFrEF) as the main feature. However, it is now clear that 50% of HF patients demonstrate symptoms of HF with preserved ejection fraction (HFpEF) [2]. It was established that HFpEF involves the progressive loss of myocardial microvascular integrity leading to an ischemia- and inflammation related fibrotic response that results to increased left ventricular stiffness [3]. While treatment options and subsequent clinical outcome have been improved over the years in patients with HFrEF, this is not true for patients with HFpEF, for whom therapeutic options remain limited [4].

Additionally, in ischemic heart disease, 20-30% of patients undergoing coronary angiography for evaluation of angina-like chest pain may suffer from non-obstructive coronary artery disease, also denoted as 'microvascular angina'. This condition is associated with low-grade chronic inflammation and coronary microvascular dysfunction [5, 6].

The worldwide increase of obesity is directly related to the prevalence of patients with type 2 diabetes mellitus (DM) [7]. These patients develop severe microvascular complications, including diabetic retinopathy, nephropathy and neuropathy [8]. Patients with diabetes and chronic renal failure also have a high risk to develop HFpEF and microvascular angina.

Microvascular involvement is also largely underestimated in patients with autoimmune disease. Systemic sclerosis (SSc) is an autoimmune disease that is characterized by microvascular damage, dysregulation of innate and adaptive immunity and fibrosis in multiple organs. The estimated prevalence ranges from 50 to 300 cases per 1 million persons and an

incidences ranging from 2.3 to 22.8 cases per 1 million persons per year [9]. The events initiating SSc are unknown. In the nailfold capillaries of SSc patients, enlarged capillaries and capillary loss can be found. This suggest that microvascular malfunction is involved in the early development of SSc [10].

The human microvessels

Mechanistically, the endothelial cells (ECs) from the microvasculature are key drivers and targets of inflammatory and thrombotic processes in microvascular diseases. They are the inner surface of the blood vessels in contact with blood and mural cells that interact with the outer surface of ECs. Mural cells most often are smooth muscle cells (SMCs) or pericytes. Larger blood vessels, in the range of 100 µm, such as the arterioles, arteries and veins, are invested with single layer of ECs, surrounding layer of SMCs, outside layer of fibroblasts and extracellular matrix (ECM) components. The microvessels, in the range of 10 µm, which are the capillaries, consist of single layer ECs, underlying basal lamina and surrounded by pericytes (Figure 1) [11, 12].

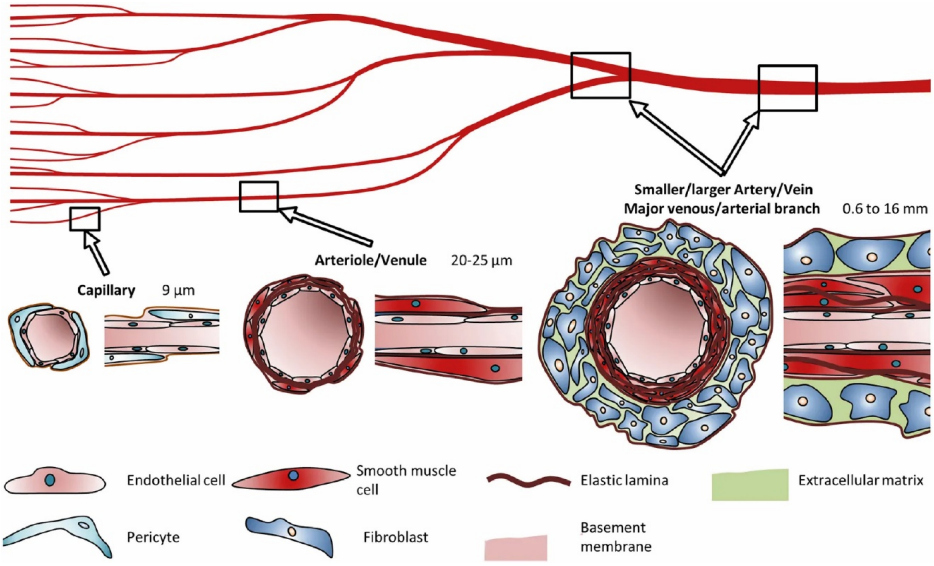

Figure 1 | Schematic of the human blood vessel. Arterioles/venules are invested with endothelial cells, smooth muscle cells and enclosed layer of fibroblasts. The capillaries, which comprise the microvessels, consists of endothelial cells surrounded by pericytes [11].

The ECs regulate blood flow, modulate coagulation, inflammation and vascular permeability in the capillaries. Destabilization of the microvessels is initiated by chronic activation of the ECs by cardiovascular risk factors that inflict the loss of pericytes, followed by the loss of EC cell-cell junctions, microvascular leakage and induction of a pro-angiogenic response [13, 14]. These processes are mediated by several angioregulatory factors, including pericyte-expressed anti-inflammatory factor Angiopoietin-1 (Ang-1) and the EC-derived pro-inflammatory factor Angiopoietin-2 (Ang-2). In normal physiological condition, Ang-1 binds to its main receptor, Tie2, that is predominantly expressed on ECs [14]. This mediates microvessel maturation and stability and reduce vascular leakage. However, in microvascular diseases such as diabetic nephropathy, Ang-2 competes for binding to the same Tie-2 receptor as Ang-1 and elicits inflammation, microvascular destabilization and leakage [15].

Insufficient diagnostic and prognostic tools for microvascular diseases

Microvascular disease has an unmet clinical need for new therapeutics, with few to no therapies available. Unfortunately, microvascular disease cannot be studied in a physiologically relevant way using static cell cultures, because current two-dimensional (2D) human models with cultured ECs lack sufficient complexity to assess the functionality of the microvascular system. Therefore, microvascular disease has been predominantly studied in experimental animal models. However, recent publications have emphasized the differences between human diseases and animal models of diseases, and how this led to failure in predicting therapeutic safety and efficacy [16]. Moreover, animal models are limited in the number of individual parameters that can be assessed at cell-type specific levels. Some have recommended abandoning animal studies and focusing more on clinical trials in patients. However, the ethic and financial burdens behind this concept is too great to overcome. Clinical trials must be conducted in agreement with ethical standards, coherent scientific proof and benefit overweigh risk [17]. On average, it takes between 10-12 years and $1.5-$3 billion to bring a new drug to the market. This is to a large extent as less than 10% of drugs that enter clinical trials end up making it to the market [18].

The use of organs-on-chips to study microvascular diseases

The development of relevant micro-physiological systems could benefit the investigation of human-specific molecular mechanisms of microvascular destabilization. The ‘organs-on-chips’ are proposed to serve as advanced micro-physiological systems that can mimic key features of microvascular homeostasis. These are biomimetic microfluidic cell culture devices with microchannels consisting of glass or silicon-based, often made with microfabrication methods that were initially designed for the electronics industry [19].

An organ-on-a-chip platform makes it possible to co-culture cells and study cell-cell interactions, such as the culture of ECs with pericytes [20]. It can provide native microvessel function in an intricate three-dimensional (3D) environment with intimate interactions between ECs and pericytes (Figure 2) [21]. Moreover, with the perfusion of whole blood or blood particles, the organs-on-chips feature laminar shear stress in order for the ECs to

maintain a quiescent anti-inflammatory phenotype. The lack of laminar shear stress in static cell cultures converts ECs to maintain a pro-inflammatory "diseased" phenotype [22]. ECs are usually cultured on surfaces such as plastics and glass that are much stiffer than ECM. Unfortunately, the cells on such hard substrate also adopt a pro-inflammatory phenotype [23]. On the contrary, in the organs-on-chips, ECs can be cultured on ECM with a modifiable physiological stiffness, forming a microvessel that is uniquely suited to study microvascular disease.

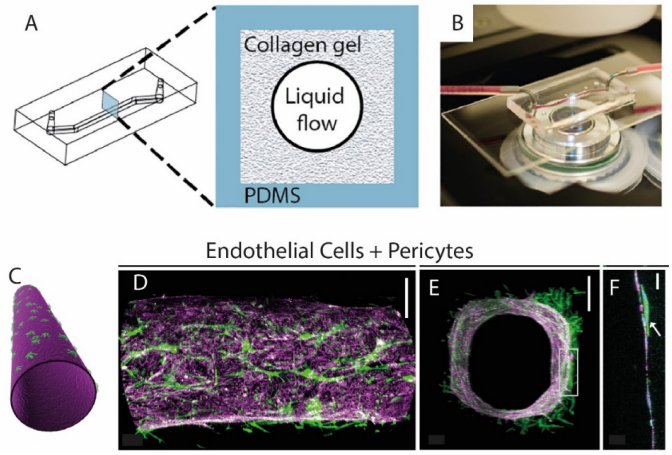

Figure 2 | The 3D human blood-brain barrier (BBB) on a chip. (A) Schematic diagram of the organ-on-a-chip showing the microchannel containing extracellular matrix (collagen) and a central lumen for generating a 3D BBB on a chip. **(B)** Photograph of the 3D BBB on a chip on the stage of a microscope. **(C-F)** The fluorescence micrographs of the brain microvascular endothelium invested with brain pericytes in surrounding extracellular matrix (bars, 200 µm in D, E and 30 µm in F). Magenta corresponds to VE-Cadherin staining of endothelial cells and green represents F-actin staining of pericytes [21].

Advancements in organs-on-chips has also enabled the production of multi-organs-on-chips. In this platform, cells are co-cultured in several organ chambers and are connected by microfluidic channels. These organ chambers can interact with each other through the flow of culture medium, simulating blood circulation. This allows investigation of pharmacokinetic and pharmacodynamic (PK/PD) parameters [24]. To fully mimic human organismal homeostasis, blood circulation through ECs-lined microfluidic channels connecting organ

equivalents with each other in a physiological order is needed [25]. In one study, a chip model was developed that connects induced pluripotent stem cells (iPSCs)-derived cardiomyocytes and hepatocytes by 3D-printed rigid filament networks of a carbohydrate glass lined with ECs and perfused with blood under high-pressure pulsatile flow [26]. This platform may serve as a unique tool for toxicology studies as well as for the development of novel therapeutic strategies to combat loss on microvascular integrity.

Various functional and biochemical read-outs can be used to gain information on the health status of the microvessel, depending on the choice of organ-on-a-chip platform. It is very common to utilize immunostaining and imaging to assess the structure and viability of the miniaturized vessel [27, 28]. In order to investigate vessel permeability or leakage, micro-structured electrodes are integrated in the microfluidic chip to measure the trans-endothelial electrical resistance (TEER) that allows continuous and non-invasive evaluation of the endothelial barrier function [29]. Another method is to quantify the diffusion of fluorescent molecules, with physiological relevant sizes, through the vessel wall [30]. This assay can be used to optimize the culture conditions for robust and long-term culture of the vessels at a low cost.

Cell metabolism in the organs-on-chips can also be used to understand the health condition of the vascular tissue model. To analyze metabolism of cells, often small molecules, i.e., metabolites and lipids such as amines and modified fatty acids are measured with metabolomics, thus providing additional information to standard end-point assays such as immunostainings, permeability assay and quantitative polymerase chain reaction (qPCR). The integration of both functional and biochemical measurements makes the read-out of the microvessel *in vitro* more accurate and informative.

With the functional and biochemical read-outs, the organ-on-a-chip platform may facilitate the identification of disease-associated circulating factors in blood that cause microvascular destabilization and help diagnosis and clinical management of patients at risk for microvascular disease related complications such as HFpEF, microvascular angina and diabetic nephropathy. It may serve to assess the functional impact of adverse circulating factors and facilitate the identification of the affected biological pathways and processes and identify potential novel therapeutic targets with sufficient efficacy and safety.

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Outline of this thesis

In recent years, a wide range of perfusable microvessel models have been developed exploiting advances in microfluidics, biomaterials and tissue engineering. Therefore, the research question that is discussed in this thesis:

How can we use a microfluidic 3D cell culture platform to develop a reliable robust and physiological relevant 'microvessel-on-a-chip' platform, that may serve as an attractive and versatile replacement for a significant fraction of animal models in vascular homeostasis?

To answer this question, the comparability of the physiology of the *in vitro* system with human situation is hereby essential. The conditions of blood plasma have to be matched and a sensitive read-out of small changes is needed. Metabolomics as read-out could be suited for this. Most importantly, the microvessel-on-a-chip should be optimally suited to model human microvessels and possess the ability to measure the presence of inflammatory leakage factors in patient-derived blood, thereby opening up the options to study loss of microvascular integrity, and ultimately allowing to perform "clinical trial on-a-chip". As microfluidic 3D cell culture platform, we have chosen the OrganoPlate because of its high-throughput capabilities [31]. This device enables the culture of endothelial cells as 96 3D and perfusable microvessels (Figure 3) [30].

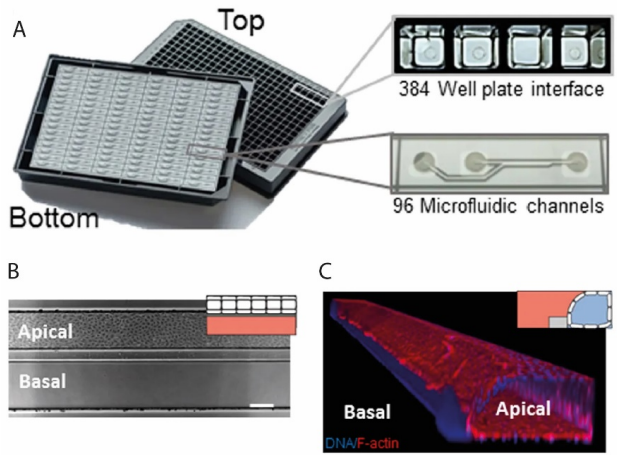

Figure 3 | Microfluidic cell culture device: OrganoPlate. (A) Photograph of bottom and top views of an OrganoPlate. The device consists of a 384 wells plate interface on top and 96 microfluidic chips integrated in the bottom. **(B)** Phase contrast image and **(C)** 3D reconstruction of a microvessel in the OrganoPlate [30].

Chapter II summarizes the current state of the art of organ-on-a-chip systems and especially evaluates the efforts put in the development of these systems in the light of end-user adoption aspects. We made an inventory of instrument compatibility, ease of handling, and adoption readiness aspects. In addition, we considered the type of assays that are typically carried out in, or on samples from, these systems, providing insight in the spectrum of techniques that can be deployed for assessing biological properties and responses, and to answer biological, clinical or pharmacological questions. The results clearly identified that the elements that bring organs-on-chips to end-users are in place, but only few systems have succeeded in applying them in order to answer biological questions.

Chapter III reports the development of a perfusable high-throughput microvessel-on-a-chip platform to study microvascular diseases, featuring a collagen hydrogel that minimally mimics the ECM. The microengineered human blood vessel allows quantitative and parallel testing of microvascular leakage. Exposure of the microvessels to VEGF, histamine and TNFα led to albumin leakage, reconstituting the microvascular leakage seen *in vivo*. Moreover, we demonstrated that this process involves changes in cellular mechanics and our findings were confirmed with TEER measurements. Especially, we developed a method to screen blood samples for vascular destabilizing factors in our device. Plasma samples were prepared from whole blood and treated with hirudin, corn trypsin inhibitor (CTI) and compstatin. Subsequently, the samples were spiked with VEGF, histamine and TNFα and perfused through the microvessels-on-chips, which showed increase in vessel permeability and confirmed the effect of destabilizing factors in blood to induce microvascular leakage in a physiological relevant *in vitro* setting. The screening of effect of plasma samples on microvessels-on-chips can be used for the development of therapeutic strategies to combat microvascular destabilization.

With the microvessels that were developed in chapter III, we show in **Chapter IV** the first chip-based model for studying mechanisms in the vascular pathophysiology associated with Ebola virus disease and provides an *in vitro* platform for drug studies. Luminal infusion of Ebola virus-like particles (VLPs) consisting of the Ebola virus matrix protein VP40 and Ebola glycoprotein ($GP_{1,2}$) leads to albumin leakage from the engineered vessels. This process involves the activation of the Rho/ROCK pathway. Moreover, VLP-induced vessel permeability is associated with changes in cellular mechanics and Ebola virus $GP_{1,2}$ in its particle associated form

mediates endothelial cell activation and increased vessel permeability. We demonstrated the utility of our platform for Ebola drug therapy by studying the recently developed experimental drug FX06 and melatonin, that strongly suppressed Ebola-induced vascular destabilization.

In **Chapter V**, we applied metabolomics to measure the metabolic state of the microvessels. To assess whether the microvessels display a less inflammatory phenotype, we used an optimized targeted liquid chromatography–tandem mass spectrometry to measure a panel of pro- and anti-inflammatory bioactive lipids to generate expression profiles in TNFα treated microvessels. We demonstrated that bioactive lipid profiles can be readily detected from the microvessels-on-a-chip. Moreover, the metabolic profile of the microvessels were compared to 2D endothelial cell cultures. The results showed that the micro-physiological system actually display a more dynamic, less inflammatory response to TNFα, that resembles more the human situation, compared to classical 2D endothelial cell cultures.

In **Chapter VI**, we developed a multi-channel microfluidic pump and *in situ* oxygen monitoring system for the microvessels-on-chips. The integrated microvessels-on-chips platform is a fully closed system and the oxygen concentration was constantly monitored in order to be sure of a constant oxygen level due to gas permeability of the tubings. The cytoskeleton remodeling of the endothelial cells under unidirectional flow with the microfluidic pump, bidirectional flow with the Mimetas perfusion rocker and static condition was quantified. Cells under static condition and bidirectional flow were more randomly orientated, whereas cells under unidirectional flow were uniformly orientated and aligned in one direction. Moreover, the microvessels under shear stress with the microfluidic pump show high expression of *Klf2* and *Klf4* compared to bidirectional flow and static condition. Our results demonstrated that the generated shear stress changes the morphology and gene expression of the microvessels-on-chips to what is found *in vivo* and deviate significantly from models without shear stress.

Finally, **Chapter VII** provides discussions and conclusions of this thesis. A critical evaluation of the research is revealed together with a discussion about the future perspective and directions of the field of organs-on-chips to study microvascular diseases.

References

1. McMurray, J.J., et al., *Clinical epidemiology of heart failure: public and private health burden.* Eur Heart J, 1998. **19 Suppl P**: p. P9-16.
2. Owan, T.E., et al., *Trends in prevalence and outcome of heart failure with preserved ejection fraction.* N Engl J Med, 2006. **355**(3): p. 251-9.
3. Paulus, W.J. and C. Tschope, *A novel paradigm for heart failure with preserved ejection fraction: comorbidities drive myocardial dysfunction and remodeling through coronary microvascular endothelial inflammation.* J Am Coll Cardiol, 2013. **62**(4): p. 263-71.
4. Cleland, J.G., P. Pellicori, and R. Dierckx, *Clinical trials in patients with heart failure and preserved left ventricular ejection fraction.* Heart Fail Clin, 2014. **10**(3): p. 511-23.
5. Lichtlen, P.R., K. Bargheer, and P. Wenzlaff, *Long-term prognosis of patients with anginalike chest pain and normal coronary angiographic findings.* J Am Coll Cardiol, 1995. **25**(5): p. 1013-8.
6. Crea, F., P.G. Camici, and C.N. Bairey Merz, *Coronary microvascular dysfunction: an update.* Eur Heart J, 2014. **35**(17): p. 1101-11.
7. Danaei, G., et al., *National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants.* Lancet, 2011. **378**(9785): p. 31-40.
8. Fowler, M.J., *Microvascular and Macrovascular Complications of Diabetes.* Clinical Diabetes, 2008. **26**(2): p. 77.
9. Gabrielli, A., E.V. Avvedimento, and T. Krieg, *Scleroderma.* New England Journal of Medicine, 2009. **360**(19): p. 1989-2003.
10. Furue, M., et al., *Pathogenesis of systemic sclerosis-current concept and emerging treatments.* Immunologic Research, 2017. **65**(4): p. 790-797.
11. Schoneberg, J., et al., *Engineering biofunctional in vitro vessel models using a multilayer bioprinting technique.* Scientific Reports, 2018. **8**.
12. Bautch, V.L., *Stem cells and the vasculature.* Nature Medicine, 2011. **17**(11): p. 1437-1443.
13. Rabelink, T.J., H.C. de Boer, and A.J. van Zonneveld, *Endothelial activation and circulating markers of endothelial activation in kidney disease.* Nature Reviews Nephrology, 2010. **6**(7): p. 404-414.
14. Armulik, A., G. Genove, and C. Betsholtz, *Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises.* Developmental Cell, 2011. **21**(2): p. 193-215.
15. Khairoun, M., et al., *Microvascular Damage in Type 1 Diabetic Patients Is Reversed in the First Year After Simultaneous Pancreas-Kidney Transplantation.* American Journal of Transplantation, 2013. **13**(5): p. 1272-1281.
16. van Esbroeck, A.C.M., et al., *Activity-based protein profiling reveals off-target proteins of the FAAH inhibitor BIA 10-2474.* Science, 2017. **356**(6342): p. 1084-1087.

17. Muthuswamy, V., *Ethical issues in clinical research.* Perspect Clin Res, 2013. **4**(1): p. 9-13.
18. Thomas, K. *The price of health: the cost of developing new medicines* 2016 [cited 2019 11-08-2019]; Available from: https://www.theguardian.com/healthcare-network/2016/mar/30/new-drugs-development-costs-pharma.
19. Novak, R., et al., *Scalable Fabrication of Stretchable, Dual Channel, Microfluidic Organ Chips.* Jove-Journal of Visualized Experiments, 2018(140).
20. Junaid, A., et al., *An end-user perspective on Organ-on-a-Chip: Assays and usability aspects.* Current Opinion in Biomedical Engineering, 2017. **1**: p. 15-22.
21. Herland, A., et al., *Distinct Contributions of Astrocytes and Pericytes to Neuroinflammation Identified in a 3D Human Blood-Brain Barrier on a Chip.* Plos One, 2016. **11**(3).
22. Baeyens, N., et al., *Endothelial fluid shear stress sensing in vascular health and disease.* J Clin Invest, 2016. **126**(3): p. 821-8.
23. Sack, K.D., M. Teran, and M.A. Nugent, *Extracellular Matrix Stiffness Controls VEGF Signaling and Processing in Endothelial Cells.* J Cell Physiol, 2016. **231**(9): p. 2026-39.
24. Zhao, Y., et al., *Multi-Organs-on-Chips: Towards Long-Term Biomedical Investigations.* Molecules, 2019. **24**(4).
25. Schimek, K., et al., *Integrating biological vasculature into a multi-organ-chip microsystem.* Lab on a Chip, 2013. **13**(18): p. 3588-3598.
26. Vunjak-Novakovic, G., et al., *HeLiVa platform: integrated heart-liver-vascular systems for drug testing in human health and disease.* Stem Cell Research & Therapy, 2013. **4**.
27. Yasotharan, S., et al., *Artery-on-a-chip platform for automated, multimodal assessment of cerebral blood vessel structure and function.* Lab Chip, 2015. **15**(12): p. 2660-9.
28. Jung, Y., et al., *Scaffold-free, Human Mesenchymal Stem Cell-Based Tissue Engineered Blood Vessels.* Sci Rep, 2015. **5**: p. 15116.
29. Kratz, S.R.A., et al., *Latest Trends in Biosensing for Microphysiological Organs-on-a-Chip and Body-on-a-Chip Systems.* Biosensors (Basel), 2019. **9**(3).
30. van Duinen, V., et al., *96 perfusable blood vessels to study vascular permeability in vitro.* Scientific Reports, 2017. **7**(1): p. 18071.
31. Trietsch, S.J., et al., *Membrane-free culture and real-time barrier integrity assessment of perfused intestinal epithelium tubes.* Nature Communications, 2017. **8**(1): p. 262.