Engineered 3D-Vessels-on-Chip to study effects of dynamic fluid flow on human induced pluripotent stem cell derived endothelial cells

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Chapter one:

General introduction

1.1 Introduction

Blood vessels make up the vascular system of the body. They are crucial for the function of all cells and tissues since they essential oxygen and nutrients and remove waste products[1]. The vascular system can be broadly divided into macro-vasculature and the micro vasculature[2].

The macrovasculature makes up the artery and venous systems and its primary function to distribute blood throughout the body. Vessels of the macrovasculature have diameters ranging from 3 cm at the aorta to 200 μm at smaller arteries and veins. The microvasculature by contrast is much smaller, 200 to 5 μm, and is made up of arterioles, capillaries and venules. This vascular-system directs blood flow to and acts as barrier between blood and individual tissues. Selectivity towards chemical compounds, electrolytes and immune cells of the tissue barriers varies from tissue-to-tissue and conditions[3]. For instance, a mature blood-brain-barrier (BBB) is a highly selective and controlled barrier that protects brain tissue from a multiplicity high risk pathogens and in general prevents immune cell and drug access[4,5]. This also means that, for example, chemotherapeutic drugs are unable to cross the BBB, making brain malignancies difficult to treat. By contrast, the kidney-blood- (with small fenestrae or pores) or liver-blood barriers (with sinusoids or large gaps) are not selective at all which allows "sieving" and rapid detoxification of the blood[6]. These phenotypic differences all contribute to sustaining organ functionality and in creating *in vitro* models of the vasculature, it is important that this is also taken into consideration.

Diseases of the vascular system and approaches to therapy

Many diseases are associated with development of aberrant vasculature, either as inducer of disease or collateral damage resulting from other insults to organ tissue. Some are introduced briefly in the following section since they form the underlying reason for building synthetic vessel-on-chip models as described in this thesis. A diseased vasculature can result in many different diseases that range from atherosclerosis[7], vascular dementia [8,9] and cancer[10], to haemorrhaging conditions that can be life-threatening[11]. Aside from chronic conditions mentioned above, diseased vasculature can also lead to a vast range of acute conditions like thrombosis[12], aneurism[13] and anaphylactic shock[14,15], that are difficult to prevent and can have severe outcomes.

One reason that it has been difficult to find therapies and the high failure-rate of drugs that do enter clinical trials is the lack of appropriate pre-clinical models to predict patient specific responses and correctly identify off-target effects[16]. Drug discovery worldwide is a multibillion-euro industry that attempts to improve the quality of life for millions of patients[17]. Although the industry has the highest standards for patient safety and drug efficacy, almost 90% of developed medicines fail in clinical trials due to unexpected

adverse effects or low efficacy [18,19]. This imposes risk to patients and loss of valuable resources and time.

In the conventional drug discovery pipeline, candidate drugs are initially extensively tested using a combination of *in vitro* cell cultures and in (humanized) animal models. The most promising then enter the clinical phase, where firstly safety testing begins on small groups of (usually young, male) volunteers (phase I, safety and feasibility) then moves to tests in small groups of patients (phase II, potential efficacy, comparing treated versus untreated groups). At each step, drugs are eliminated and only a small group reach phase III (efficacy in a large, randomized population of patient).

Drug responses are often the result of complex multicellular interplay, the drug delivery route (e.g. intravenous, oral) and metabolic processes for example in the liver contributing to the net effect[20,21]. Even for drugs that reach the market, only 1 in 10 patients will respond to widely prescribed drugs[22]. For this reason, the field of pharmacogenetics demonstrated the need for precision and personalized medicine[23].

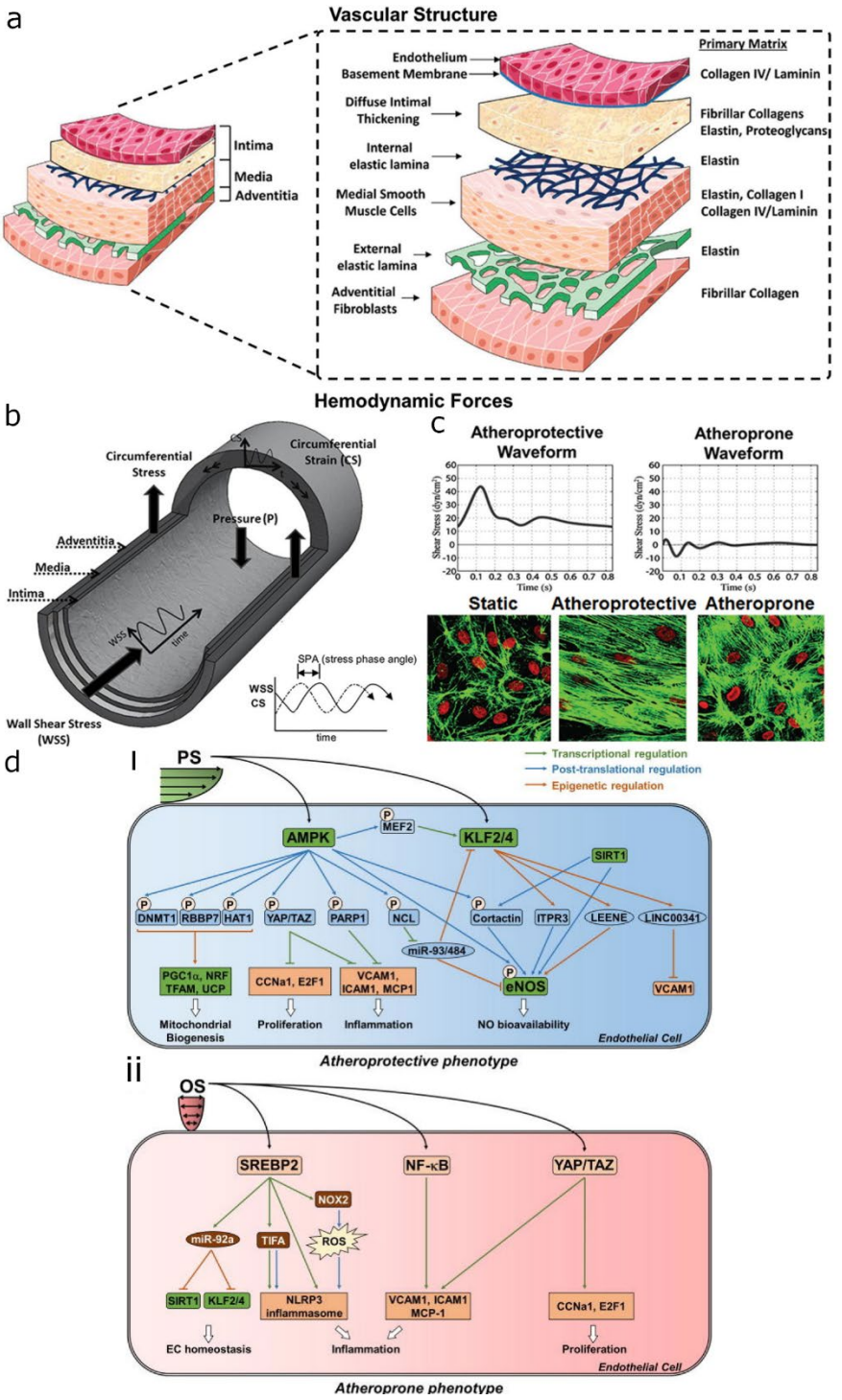
Animal models are useful to test drugs and gain more insight into drug distribution and clearance in a complex system but due in part to interspecies differences, direct translation to humans is often not possible or not relevant for all patients[24-27]. In addition, extensive use of animal models except where absolutely essential is ethically under debate due to animal welfare considerations and legislation in many jurisdictions already requires reduction, refinement and replacement of animal models with ethically acceptable alternatives[28,29]

Blood Vessel Anatomy

A blood vessel is composed out of three distinct layers (Figure 1a). The internal vessel wall, or *tunica intima*, is lined with a single layer of Endothelial cells (ECs). The ECs form the barrier between blood and tissue. ECs are phenotypically highly heterogeneous throughout the (human) body and this heterogeneity is controlled by many environmental factors, from surrounding tissue type and growth factors to oxygen levels or mechanical stimuli[30].

ECs are surrounded by the *tunica media* which is composed of a basement membrane and pericytes or smooth muscle cells, referred to as mural cells. The mural cells mechanically support ECs and allow efficient blood flow-distribution by contracting and dilating. This changes the vessel diameter and can direct blood according to tissue demands.

The macrovasculature also has an additional layer, the *tunica adventitia*, to support the vessels whereas the microvasculature is embedded inside the organs.


a
Vascular Structure
Intima
Media
Adventitia
Endothelium
Basement Membrane
Diffuse Intimal Thickening
Internal elastic lamina
Medial Smooth Muscle Cells
External elastic lamina
Adventitial Fibroblasts
Primary Matrix
Collagen IV/ Laminin
Fibrillar Collagens Elastin, Proteoglycans
Elastin
Elastin, Collagen I Collagen IV/Laminin
Elastin
Fibrillar Collagen
b
Hemodynamic Forces
Circumferential Stress
Circumferential Strain (CS)
Pressure (P)
Adventitia
Media
Intima
WSS
time
Wall Shear Stress (WSS)
SPA (stress phase angle)
WSS
CS
time
c
Atheroprotective Waveform
Atheroprone Waveform
Shear Stress (dyn/cm²)
Time (s)
Static
Atheroprotective
Atheroprone
d
I
PS
Transcriptional regulation
Post-translational regulation
Epigenetic regulation
AMPK
MEF2
KLF2/4
SIRT1
DNMT1
RBBP7
HAT1
YAP/TAZ
PARP1
NCL
Cortactin
ITPR3
LEENE
LINC00341
miR-93/484
PGC1α, NRF TFAM, UCP
CCNa1, E2F1
VCAM1, ICAM1, MCP1
eNOS
VCAM1
Mitochondrial Biogenesis
Proliferation
Inflammation
NO bioavailability
Endothelial Cell
Atheroprotective phenotype
ii
OS
SREBP2
NF-κB
YAP/TAZ
NOX2
miR-92a
TIFA
ROS
SIRT1
KLF2/4
NLRP3 inflammasome
VCAM1, ICAM1 MCP-1
CCNa1, E2F1
EC homeostasis
Inflammation
Proliferation
Endothelial Cell
Atheroprone phenotype

Vascular phenotype

As mentioned earlier, the EC-phenotype is highly heterogenic across the different organs[31]. ECs form a tissue-specific-barrier between circulating blood and the tissue itself that is vital for oxygen and nutrient delivery, waste removal and bidirectional immune-cell trafficking[32]. For instance, in addition to controlling selective transport the BBB maintains glucose and ion gradients to support high neuronal activity[33]. This contrasts with the non-specific blood barrier of the liver and kidney that allows fast detoxification of the body[36]. These different EC-phenotypes are essential for maintaining organ-homeostasis and because of their different barrier-functions, blood vessels are a major determinant of the absorption, distribution, metabolism excretion (ADME) of drugs and thus determine effective drug doses[37,38].

This EC-phenotype tissue specificity is highly plastic and constantly changing under influence of many biological cues that include not only the surrounding tissue cells but also the extracellular matrix (ECM), fluid flow rate and drugs[30,39,40].

Haemodynamics are essential pathway modulators

Haemodynamic forces like wall shear stress (WSS) and circumferential stress (CS) are important modulators of cell signal transduction (Figure 1b and c)[35,41-44]. For instance, different WSS-profiles directly influence the EC-mural cell interactions. WSS is the force parallel to the flow direction exerted on the vessel wall. ECs have multiple different molecular sensors and pathways that can directly detect haemodynamic forces and react to any local differences like primary ciliary[45], Piezo-1[46-48], integrins[49], cellular junctions[50].

WSS can have different waveforms for example; unidirectional or oscillatory. Inside an artery an unidirectional flow is considered an athero-protective waveform whereas oscillatory flow is athero-prone for the EC-phenotype (Figure 1b and c)[51]. These different waveforms directly control the EC- signal transduction (Figure 1 d). As these different flow patterns modulate important nodes of other signalling pathways, of for instance the TNF-alpha transduction, haemodynamic forces may have major implications in the inflammation response[52,53]. In standard, static *in vitro* cell culture, exposure of ECs to TNF-alpha results in loss of cell junctions, increasing leucocyte adhesion and apoptosis. When cells are exposed to TNF-alpha combined with unidirectional WSS, the subsequent complex signal transduction pathways result in an opposite, anti-inflammatory responses

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***Figure 1: Vascular structure and haemodynamic forces**. **(a)** blood vessels typically consist of 3 distinct cellular layers separated by a layer of ECM. **(b)** Important haemodynamic forces are WSS and circumferential stress **(c)** different wavefronts result in different cellular morphology.**(d)** different wavefronts are important modulators of cellular transduction. Unidirectional flow results in an athero-protective phenotype as bidirectional flow can result in an atheroprone phenotype. Figure Panels a, b and c adapted from Pradhan et. al[34] Panel d adapted from He et al.[35]*

These differences illustrate the need to account for biomechanical forces in considering drug responses both *in vivo* and *in vitro* models.

Cells used in current *in vitro* models

Broadly speaking, immortalized cell lines or primary cells are used as a basis for developing human models of disease and in early drug screening platforms.

Primary cells derived from donors and generally retain a tissue specific phenotype for a certain time in culture. However, they have a limited capacity for expansion in culture and quickly reach senescence so that eventually only relatively few experiments can be carried out with one cell batch[56]. Donor-to-donor variation and reproducibility of primary cell experiments means that considerable effort is required to create large cell batches or compare new batches with a master cell bank. Furthermore, primary cells can lose important traits when in cell culture, essentially de-differentiating, in part due to missing biochemical and biomechanical signals in their natural environment or 2D culture conditions on hard (plastic) tissue culture substrate[57].

Immortalized cell lines (either derived from tumors or created by transformation of primary cells in culture through, for example, expression of oncogenes or induction of telomerase activity) bypass cellular senescence and thus can easily be expanded for experimental purposes virtually indefinitely[58-60]. However, although immortalized cells may retain some cell specific features, important phenotypic traits can be lost or altered during prolonged passage e.g. through genetic drift or acquisition of *de novo* mutations, influencing experimental results[61]. These changes in genotype and karyotype limits their use as *bon fide* models and an entirely representative of their tissue of origin[62-64].

Human induced pluripotent stem cells (hiPSC) as *in vitro* models

Human induced pluripotent stem cells (hiPSC) are demonstrating increasing potential as valuable cell sources for drug discovery and disease modelling (Figure 2) [65]. hiPSC can be derived from virtually every healthy individual or patient by reprogramming somatic cells through the introduction of four transcription factors[66]. Different cell types can be used as a somatic cell source including fibroblasts, peripheral blood cells[68], hair follicles[69] or even (kidney)cells that can be collected from urine[70] . These non-invasive methods allow the easy generation of hiPSC lines containing the complete genotype of the individual from whom they were derived. This allows testing of drug effects or building of disease models on a wider range of genetic backgrounds (SNP-variations) prior to clinical studies. Second, hiPSCs are capable of differentiating to all cell types of the human body by administering growth factors, small molecules or ECM in sequences that mimic that in embryonic development.

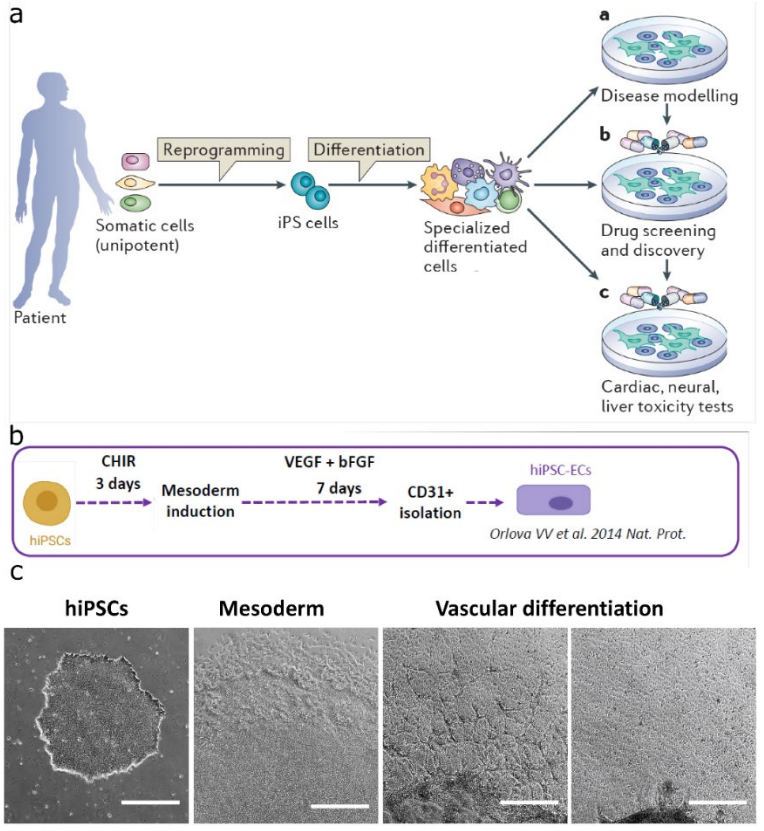
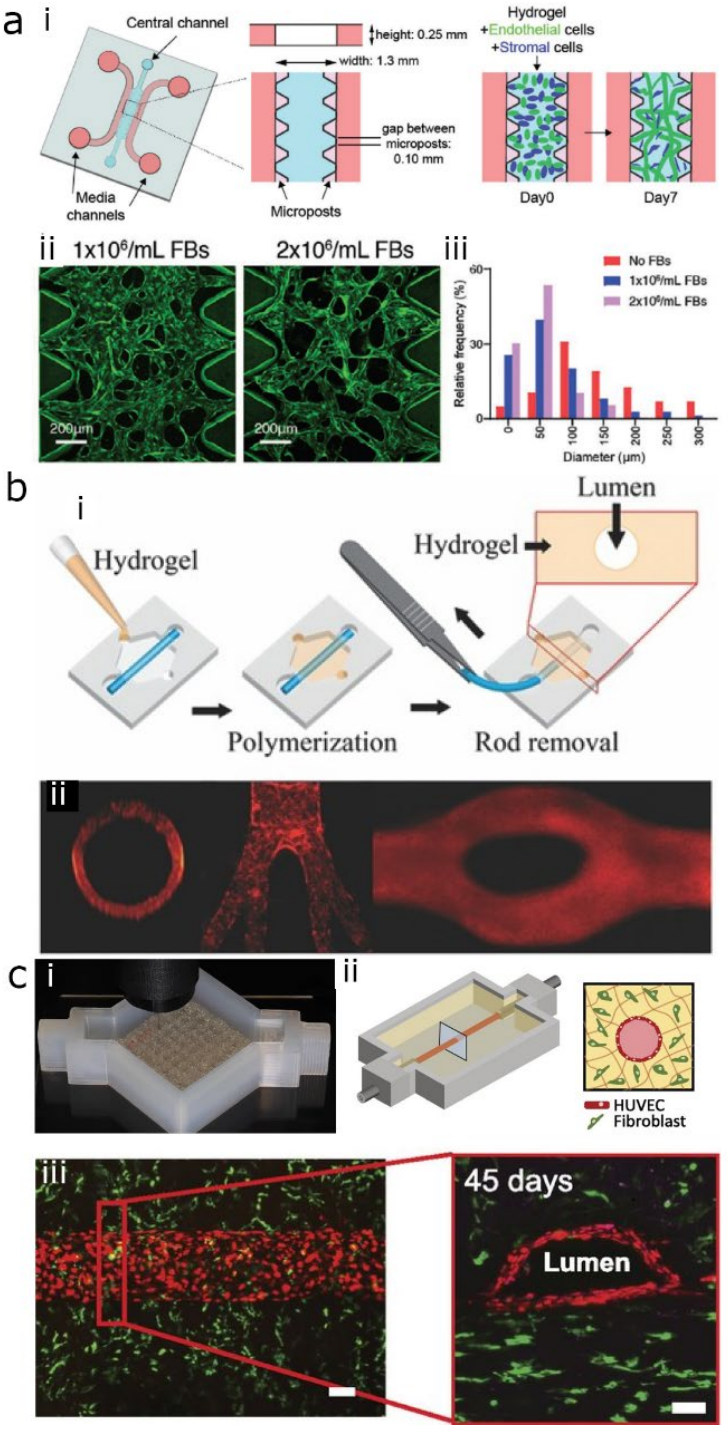


Figure 2: hiPSCs and vascular differentiation. (a) *hiPSC can be derived from virtually any patient by reprogramming somatic cells. These pluripotent stem cells can be then differentiated to specialized cells which can be used for either disease modelling, drug discovery and toxicity tests. Figure adapted from Bellin et al. (2012)[65]* ***(b)*** *Differentiation protocol from hiPSC towards endothelial cells Figure adapted from Marc Vila Cuenca,* ***(c)*** *Widefield images show morphology changes of hiPSCs towards endothelial cells. Figure adapted from Orlova et al (2014)[67]*

This allows for the generation of cell types that are difficult to harvest from primary tissue such as neurons and cardiomyocytes because biopsies for these tissues are highly invasive. Thirdly, in the case of genetic diseases caused by known mutations, it is possible to repair the gene of interest using genome editing techniques, particularly CRISPR/Cas9, and generate isogenic controls for comparison without the mutation and also to create isogenic co-cultures where greater *in vitro* tissue complexity is required for the model[71].

Finally, hiPSC have self-renewal capabilities and, when properly maintained, form an unlimited source of undifferentiated cells or banked differentiated cells with less batch-to-batch variation as compared to primary cells, allowing batch-to-batch reproducibility to be measured and more robust experiments. Genetic modification using Crispr/Cas9 techniques also allows the generation


a
i
Central channel
height: 0.25 mm
width: 1.3 mm
gap between microposts: 0.10 mm
Media channels
Microposts
Hydrogel
+Endothelial cells
+Stromal cells
Day0
Day7
ii
1x10⁶/mL FBs
2x10⁶/mL FBs
200μm
200μm
iii
No FBs
1x10⁶/mL FBs
2x10⁶/mL FBs
Relative frequency (%)
Diameter (μm)
b
i
Hydrogel
Polymerization
Hydrogel
Lumen
Rod removal
ii
c
i
ii
HUVEC
Fibroblast
iii
45 days
Lumen

of genetically encoded reporter lines that can be used for various experimental outputs including monitoring changes in structural marker proteins or cellular parameters like calcium flux or membrane potential [72-74].

In vitro differentiation of endothelial cell from hiPSCs starts by inducing mesoderm formation followed by endothelial specification (Figure. 2b) [67]. These differentiation protocols take 10 days and by cell selection yield cryopreserved-vials of pure, isogeneic batches of hiPSC-derived ECs ideal for assay development.

Although hiPSC derived ECs have many advantages over primary and immortalized cell lines, they also have disadvantages. hiPSC derivatives are generally considered "immature", meaning the cells may lack a complete adult organ-specific phenotype. The maturity of the EC-phenotype can be improved by modifying differentiation[75], co-culturing with tissue specific cells[76] and the exposure to haemodynamic forces[77]. The following section describes a number of engineered systems that have been examined towards this goal of improving phenotypic realism and increasing maturation state of the hiPSC-derived vessels.

Organ on chip

Microphysiological systems (MPS) or "Organs-on-a-Chip" (OoC), are being developed to combine different biological cues and biomechanical stimuli to promote mature cellular responses *in vitro* that resemble those *in vivo*[78]. Different OoCs are being developed form different organs: Heart-on-Chip, Gut-on-Chip, and Brain-on-Chip are already advanced models for this purpose and are increasingly used to investigate aspects of human physiology. Different OoCs can also be combined to investigate how different organs influence each other[79].

3D-Vessel on chips

As the vasculature is a vital component of every organ, different types of Vessel-on-Chips (VoCs) and Organ-barriers are being developed to mimic for example the BBB [80,81], glomerular -barrier[82,83], alveolar-barrier [84-86] and the placenta-barrier on-Chip[87]. Many methods are being used for VoCs to combine different tissue-specific cell types with multiple vascular cell types and include haemodynamic forces like those *in vivo*. These methods include self-assembly or self-organization of the vascular cells in various types of

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Figure 3: Common methodologies to engineer 3D-VoCs. (a)(i) *Cellular self-assembly comprises of mixing vascular and tissue specific cells with ECM.* ***(ii)(iii)*** *network formation is depending on cellular composition adapted from Wan et al. (2022)[88].* ***(b) (i)****Template casting comprises of casting ECM around a tubular template****.(ii)*** *cell can be seeded in the resulting lumen. Figure adapted from Jimenz-torres et al (2016)[90](c)(i)(ii) Bioprinting allows the formation of complex 3D structures****(iii)*** *multiple cell types can be cultured for extended periods of time, however shape of cross-sectional flow fields depend on bio-ink parameters, figure adapted from Kolesky et al (2016)[93]*

chambers or devices[80,88,89], template "casting"[90-92] and 3D bioprinting[93]. Each of these methods has their advantages and disadvantages and different approaches are used to investigate different aspects of vascular function or development. Cellular self-assembly has been a widely adopted methodology where ECs are mixed with ECM and allowed to form a 3D capillary bed autonomously (Figure 3a)[89]. Different cell types can be combined in different ECMs to model for example a microvascular BBB[80]. However, these self-assembled capillary beds, have one major limitation: there is no spatial control of the capillary bed and therefore all samples have different geometries and diameters. This variation makes it difficult to introduce uniform haemodynamic forces as every cell in the systems experiences these differently depending on their position and the diameter of the vessel in which they are situated[94]. To include controlled haemodynamics forces, scaffolds can be engineered with more defined geometries. Cells can be seeded into these scaffolds in any combination and density. The scaffolds can be fabricated for example using template casting[90] or 3D bioprinting[93,95](Figure 3b and c). By simply casting a hydrogel around a tube-like template, like a hypodermic needle, scaffolds can be engineered that are suitable quickly and easily suitable for cell seeding. 3D-bioprinting allows the engineering of higher order structures which can also be used to model the complex flow patterns observed for instance at vascular bifurcations . Although these methods allow the inclusion of all aspects of the vasculature, the vessels in the models are typically larger than *in vivo*[96] which could impact how cells interact with each other and how scaling of the haemodynamic forces and metabolic parameters is carried out[97].

Current limitations of VoC models

Although many methods support the generation of complex 3D vascular models, their adoption is still rather limited to biomedical research. This is because many methods have been developed as proof-of-concept but not with the intention to be low tech and easy to implement. Adoption by non-specialist labs is often limited: methodologies are complex, not scalable, require specialised equipment and expertise or the reagents and tools are expensive[98].

1.2 Aim and scope of this Thesis

To improve the predictive power of *in vitro* models, vascular biology needs to be integrated into functional and preferably facile technology. This thesis describes the development of 3D microfluidic blood Vessel-on-Chip models that can be used for applications such as disease modelling and drug discovery. The cellular components of the VoCs are derived from hiPSCs and consist of vascular ECs and pericytes. The methods were developed to include adjustable microfluidic flow that can mimic haemodynamic forces of different blood vessels with different diameters and the option to incorporate additional tissue specific cells, which expands the flexibility of the technology.

The overall aim is to improve cellular responses such that it more closely resembles that of vascular cells in blood vessels in health and disease.

In **Chapter** 2, a method is described on to generate 3D VoCs suitable for hiPSC-derived vascular cells. It optimizes an existing protocol that employs a microfluidic technique called viscous finger patterning (VFP) and it overcomes two mayor limitations of current techniques: it is a scalable method that does not require the use of highly specialized equipment or expertise and it is capable of generating uniform haemodynamic forces within the vascular architectures over extended lengths that to date have not been achieved.

Chapter 3 describes a custom PID-controller that controls the perfusion of microfluidic OoCs. This controller is essential for the perfusion and recirculation of the developed fluidic circuit board. It represents and advance on current software control of this type of system.

In **Chapter 4** a multiplexing Fluidic Circuit Board (FCB) is described that is capable of generating equal wall shear stress in parallel to 3D-VoCs with intrinsic diameter variation. The FCB also allows the control of circumferential stress the 3D-VoCs which can be used to model different (patho-) physiological conditions.

Chapter 5 describes on the development of a realistically-sized vascular capillary network model. The methodology combines mask-less photolithography techniques and cellular self-assembly to engineer true 3D vascular capillary mimics.

Together, the research described in this thesis, provides significant practical and theoretical advances that can improve the utility of VoC systems to biological laboratories. This type of multidisciplinary link between device engineering and testing cells increasingly used in biomedical research because they are representative human disease models, will contribute to more widespread adoption of OoC technology. This is considered in more depth in the General Discussion in **Chapter 6**.

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