UNDERSTANDING WET AGE- RELATED MACULAR DEGENERATION

**AN ORGAN-ON-A-CHIP MODEL OF THE OUTER
BLOOD-RETINAL BARRIER**

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The research presented in this thesis was carried out at the department of Applied Stem Cell Technologies at the TechMed Centre for Biomedical Technology and Technical Medicine in collaboration with the BIOS - Lab on a Chip group at the MESA+ Institute for Nanotechnology at the University of Twente, Enschede, The Netherlands. Financial support was received from the Toegepast Wetenschappelijk Instituut voor Neuromodulatie (TWIN) under the project name "Inflammation and Edema in an Organ-on-a-Chip Model of Wet Age-related Macular Degeneration" coordinated by Dr. Andries D. van der Meer

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UNDERSTANDING WET AGE- RELATED MACULAR DEGENERATION

**AN ORGAN-ON-A-CHIP MODEL OF THE OUTER
BLOOD-RETINAL BARRIER**

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
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on account of the decision of the Doctorate Board,
to be publicly defended
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1

Introduction

In this chapter, the framework, background information and motivation for the thesis are presented. In the last section, an outline of the thesis chapters is given.

1.1 Framework and Aim of Thesis

The research described in this thesis was funded by Toegepast Wetenschappelijk Instituut voor Neuromodulatie (TWIN) under the project name “Inflammation and Edema in an Organ-on-a-Chip (OOC) Model of Wet Age-related Macular Degeneration” (wet-AMD) coordinated by Dr. Andries van der Meer. The purpose of this grant was to create an *in vitro* microfluidic OOC platform of the outer blood retinal barrier (oBRB) for the investigation of the current leading cause of blindness in elderly, AMD, and in particular, its most aggressive form, wet-AMD.

This thesis aims to report on developing this platform that recapitulates the native *in vivo* microenvironment of the oBRB with clinically relevant readouts to validate the disease conditions. We believe that this will pave the way for developing and testing new treatments for AMD. Currently, AMD therapy involves series of treatments that are expensive and stressing for patients. Anecdotal evidence in patients suggests that two widely accessible anti-inflammatory (aescine) and anti-histaminic (cetirizine) drugs also work. About 10 patients used these oral compounds daily and at least 8 of them claim to have beneficial effects on their eyesight. The visible changes to their life quality can be experienced as early as 6 months following the regular administration of these drugs. However, in order to prove the effectiveness of these treatments properly, extensive clinical trials are needed. Clinicians are only willing to undertake such trials if the presumed mechanism of action of this experimental treatment can first be further demonstrated and proven. The platform presented in this thesis aims to pave the way for further clinical studies and more widespread clinical and pharmaceutical acceptance of this promising treatment for wet-AMD.

The research for this thesis was performed at the department of Applied Stem Cell Technologies at the TechMed Centre for Biomedical Technology and Technical Medicine in collaboration with the BIOS/Lab on a Chip group of the MESA+ Institute for Nanotechnology at the University of Twente in Enschede, the Netherlands under the supervision of Dr. Andries van der Meer, Prof. Dr. Robert Passier and Prof Dr. Ir. Albert van den Berg.

1.2 Background and Motivation

Visual impairment significantly affects life quality of patients, rendering them unable to perform simple everyday tasks such as reading, recognizing faces, driving etc. Visual impairment and blindness are already major direct and indirect burdens on healthcare which cost 3 trillion USD on estimate in 2010 annually.^{1,2} This burden will only increase in the coming decades as the global population ages.³ Among blindness, AMD is the third major cause of blindness and accounts for 8.7% of global cases⁴ and in western societies, it is currently the most common cause of blindness in people over 60.^{5,6} A report in 2014 estimated the number of AMD patients to be 196 million by 2020 and increasing to 288 million in 2040.⁴

The key tissue that is affected in AMD is the macula. The macula is a specialized region in the retina with the highest metabolic demands, as it is responsible for the sharpest, central vision of our visual acuity.⁷ Due to its high metabolism, it is prone to dysfunction through decades of aging. Waste disposal and nutrient exchange of photoreceptors in this specialized

region are maintained by retinal pigment epithelium and the surrounding choroidal capillaries (Fig.1.1A).

It is not certain which series of exact events lead to the initiation and progression of AMD. However, it is multi-factorial where genetic and environmental factors are in play. Its most aggressive form, wet-AMD, is characterized by choroidal neovascularization (CNV), in which new blood vessels invade the normal tissue barriers of the outer retina from the underlying choroid bed of blood vessels (Fig.1.1B).⁷ These vessels easily hemorrhage and eventually leak their contents below and within the retina, which leads to degeneration of photoreceptors in the macula (Fig.1.1C). This causes a rapid vision loss of central vision. Vision loss occurs due to a combination of stressors where toxicity to the cells accumulate over time which systemic repair mechanisms fail to heal.

Figure 1.1 Schematics of outer retina and adjacent choroidal vessel network. (A) Healthy retina with intact photoreceptors, retinal pigment epithelium, Bruch's membrane, and choroidal vessels. (B) Early signs of dry AMD is characterized by the accumulation of insoluble deposits called 'drusen' below or within outer retina. (C) Early stage of AMD can transform into severe/ wet-AMD characterized by the ingrowth of blood vessels into the retina. Due to fluid accumulation from the hemorrhaged vessels, photoreceptors are rapidly damaged, causing severe vision loss.⁸

To be able to understand the pathophysiology of AMD several animal models have been developed. However, these models suffer from specific issues such as interspecies anatomical differences, requiring long durations for disease progression, high costs, and ethical issues. On the other hand, most of the current laboratory models based on human tissues involve simple monolayers of cells, which fail to mimic the 3D, tissue-level physiology.^{9,10} To better understand the disease pathology of AMD, experimental models are required in which the morphological changes of the tissues can easily be observed and experimental conditions can be readily manipulated.

In recent decades, thanks to microfluidics and microfabrication technologies, *in vitro* modelling platforms evolved into OOC devices. These cell culturing devices comprise individually perfused microchannels inhabited by living cells to create a realistic simulation of a tissue or organ of interest.¹¹ Co-cultures of cells are usually separated by porous membranes to allow cross-talk between different cell types.¹¹ This thesis will demonstrate that OOCs may offer unique opportunities to model AMD and to study new potential treatments for this debilitating disease.

1.3 Thesis Outline

As described above, loss of barrier structure in the retina is a central aspect of the mechanism of AMD. Therefore, the tools available to evaluate barrier function of tissues in cell culture models is described in more detail in **Chapter 2**. This review chapter lists various techniques for measuring permeability in conventional platforms as well as OOC devices. At the end, it provides an overview of different OOC models that measure permeability. **Chapter 3** presents the design, development and evaluation of an OOC device that models the oBRB. This device contains a microchannel where a collagen-based hydrogel is patterned into a microvessel inhabited by human umbilical vein endothelial cells, an open-top culture chamber where retinal pigment epithelial cells (RPE) are cultured as a monolayer and a polyester membrane separating these co-cultures. Permeability of the microvessels and RPE are tracked using fluorescent tracers. Optical coherence tomography was implemented to confirm sizes and structural changes of the engineered microvessels. Using this model, we investigated one of the earlier hallmarks of wet AMD: the increased permeability of blood vessels due to reactive oxygen species exposure. **Chapter 4** addresses the limitation of including plastic porous membranes in our OOC of the oBRB and OOC in general. Such membranes are typically made from synthetic and biologically inert materials. In this chapter, an innovation is presented in the form of a vitrified membrane made of collagen-I hydrogel which is integrated in an OOC device. Biocompatibility and bioactivity of these membranes was confirmed by cell culture and enzymatic degradation experiments. **Chapter 5** discusses the presumed mechanism of anti-inflammatory and anti-histaminic drugs against the effects of TNF- α and histamine using the changed morphology of endothelial cells as a readout. Finally, in **Chapter 6**, a general discussion of the results from this thesis and future perspectives are given.

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2

Barriers-on-Chips: Measurement of Barrier Function of Tissues in Organs-on-Chips

Disruption of tissue barriers formed by cells is an integral part of the pathophysiology of many diseases. Therefore, a thorough understanding of tissue barrier function is essential when studying the causes and mechanisms of disease as well as when developing novel treatments. *In vitro* methods play an integral role in understanding tissue barrier function, and several techniques have been developed in order to evaluate barrier integrity of cultured cell layers. This ranges from microscopy imaging of cell-cell adhesion proteins to measuring ionic currents, to flux of water or transport of molecules across cellular barriers. Unfortunately, many of the current *in vitro* methods suffer from not fully recapitulating the microenvironment of tissues and organs. Recently, organ-on-a-chip (OOC) devices have emerged to overcome this challenge. OOCs are microfluidic cell culture devices with continuously perfused microchannels inhabited by living cells. Freedom of changing the design of device architecture offers the opportunity of recapitulating the *in vivo* physiological environment while measuring barrier function. Assessment of barriers in OOCs can be challenging as they may require dedicated setups and have smaller volumes that are more sensitive to environmental conditions. But they do provide the option of continuous, non-invasive sensing of barrier quality, which enables better investigation of important aspects of pathophysiology, biological processes and development of therapies that target barrier tissues. Here, we discuss several techniques to assess barrier function of tissues in OOCs, highlighting advantages and technical challenges.

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2.1 Introduction

The human body contains numerous barriers, some of which separate the internal environment from the external environment and others that separate different compartments inside the body. These barriers are found in for example skin, airways, brain, eye, and blood vessels, and they maintain homeostasis by regulating the interactions between the compartments that they separate. Moreover, barriers such as the blood-brain barrier (BBB), blood retinal barrier (BRB) and the pulmonary air-liquid interface (ALI) are highly selective to prevent toxins from affecting vital organs. Disruption and dysfunction of such tissues are of major importance in the pathophysiology of many human diseases (e.g. BBB disruption in multiple sclerosis, meningitis, encephalitis ¹, BRB disruption in diabetic retinopathy, macular degeneration ², ALI disruption in pulmonary edema ³).

It is well-known that the biophysical and biochemical tissue microenvironment in terms of blood flow, interstitial flow, tissue shape and curvature, mechanical strain, paracrine signaling, and the local interaction between various cell types all play important roles in maintaining or altering barrier function of tissues. ⁴⁻⁷ Current *in vitro* methods fail to provide this dynamic physicochemical microenvironment. Therefore, there is a strong need for advanced *in vitro* systems that allow the controlled and routine inclusion of a realistic tissue microenvironment when studying the barrier function of cultured cells.

OOCs are a new class of microphysiological *in vitro* models of human organs and tissues that rely on culturing cells in a well-controlled microenvironment that has been engineered to include key physical and biochemical parameters ^{5,8-14}. OOCs show great promise in mimicking human tissues and organs and are being used in both fundamental and translational biomedical research. For OOCs to be valuable as research tools, it is essential that the state of the cells in an OOC can be probed and quantified in various ways. Some of the most often measured physiological parameters in the current generation of OOCs are related to tissue barrier function. Importantly, measuring permeability in OOCs is associated with unique challenges that are related to their small size, low volumes and dynamic nature. It is essential to understand these challenges and to analytically characterize the OOC system that is being used.

In this chapter, we give examples of OOC systems in which various parameters related to barrier function were routinely measured. Since this chapter is based on a review article published in 2018, examples until this date has been highlighted. Furthermore, we discuss the advantages and challenges of measuring barrier function in OOC systems and we give practical pointers for avoiding the most common measurement errors. Although active receptor-mediated transport is very important in physiology and drug discovery, and OOC systems show great promise in realistically mimicking physiological expression profiles of receptors ¹⁵⁻¹⁷, active transportation of molecules will not be discussed in this chapter. The assessment of cellular active transport *in vitro* has been discussed elsewhere ¹⁸, and the same is true for the potential role of OOC in drug discovery ¹⁹.

2.2 Cell Culturing Platforms for Barrier Assessment

Prior to giving examples and information about the methods to quantify barrier integrity in OOCs, the following section gives an overview of methods in conventional *in vitro* models

which commonly use Transwell systems. Since fundamental principles of these techniques are similar in different platforms, basics discussed below will help to understand the techniques in OOC platforms.

2.2.1 Conventional Cell Culturing Systems

Because barriers are so important in health and disease, experimental *in vitro* tools that can be used to quantify and characterize the barrier function of cells and tissues are widely used. Most conventional techniques typically make use of a Transwell cell culture system, which relies on a tissue-culture plate with two culture compartments – the well and the insert – that are separated by a synthetic porous membrane (Fig.2.1). When cells are grown on the synthetic membrane, their barrier function can be assessed by measuring various parameters. In addition to assessing barriers by imaging cell-cell junction proteins using fluorescent and electron microscopy, there are various parameters that can be measured to evaluate the barrier function of cultured cell layers: electrical resistance, mass transport and hydraulic conductivity; all three parameters will be discussed briefly below.

Transepithelial/endothelial electrical resistance (TEER) is one of the widely used methods for evaluating barriers; it gives an indication of the tightness of cell-cell junctions in the paracellular space by means of electrical resistance across a monolayer. For measurements, a commercially available Epithelial Voltohmmeter (EVOM) is often used which consists of a pair of legs with two pairs of electrodes. One of these legs is placed in the upper compartment whereas the other is submerged into the culture medium in the lower compartment (Fig.2.1A). Each of these legs contains an electrode to apply a current to the barrier, while the other electrode of the pair is used to measure the resulting voltage over the barrier. Since direct current can be damaging to the cells and electrodes, an alternating current of 10 μ A amplitude with a square waveform and a relatively low frequency (typically 12.5Hz) is applied. For analyzing TEER, first the resistance of the permeable membrane only (without cells, R_{membrane}) is measured, followed by a measurement of the resistance across the cell layer on the membrane (R_{total}). The specific resistance of the cell layer (R_{cells}) is then calculated by subtracting the blank resistance from the total resistance (Equation 1) and normalizing for cell culture area (A_{membrane}) (Equation 2.2) ²⁰

$$R_{\text{cells}} = R_{\text{total}} - R_{\text{membrane}} \quad (2.1)$$

$$TEER_{\text{cells}} = R_{\text{cells}} \times A_{\text{membrane}} \quad (2.2)$$

Readings of TEER are highly dependent on the electrode positions, and careful handling while placing the electrodes is important as it might disturb the cell monolayer. In addition, having a uniform current density generated by the electrodes has an impact on TEER values. To accurately provide that, correct type of electrode systems should be chosen. For instance, classical STX2/Chopstick electrode cannot be used for relatively large membrane (i.e. 24 mm diameter) in tissue culture inserts. ²¹ This may result in overestimation of TEER value, and as an alternative in this case, a better suited EndOhm chambers can be used to cover larger areas. ²² TEER is a sensitive, non-invasive method and with dedicated measurement systems, it is possible to monitor live cells during various stages of growth, differentiation or experimental treatment.

Barrier integrity of cells can also be assessed by measuring the paracellular diffusive transport of tracer compounds of various molecular weights. While TEER measures the ion flux through the barrier, studies of paracellular transport can give more detailed information

about the paracellular spacing when using different tracers of defined molecular weights. Tracers are typically added to the insert, and their diffusion over the cell layer into the well is tracked over time to determine the molecular flux (Fig.2.1B). Tracers can be radioactively, fluorescently, or enzymatically labeled. Radiolabeling is capable of detecting subtle changes in a barrier, however, they require special handling and safety measures, and their short half-life means they cannot be stored for long periods. Therefore, this type of labeling is not usually preferred for barrier assessment.²³ On the other hand, usage of enzymatic markers (e.g. horseradish peroxidase) has been reported for macromolecular diffusion. Low amounts of enzymes can still be sensitively quantified with the addition of sufficient amount of substrate and spectroscopically measuring the product of the catalyzed reaction, but the activity of enzymes can be affected by factors such as pH, temperature and serum constituents thereby limiting its application.²⁴ Due to ease of handling, non-radioactive, fluorescently labeled marker polysaccharides (e.g. fluorescein isothiocyanate (FITC)-labeled dextran) or proteins are widely used for permeability assays.²⁵ Depending on the biological application, the size of tracer compounds can vary widely (e.g. inulin (5 kDa), mannitol (182 Da), albumin (67 kDa)).²⁰ Despite ease-of-use, fluorescent tracers sometimes lack the required sensitivity to detect subtle changes in barriers due to poor specific activity (fluorescence/mg protein) or fluorophore instability.²⁴ In general, it should be noted that the use of any tracer compounds may interfere with the transport process under study and may affect the barrier integrity as well as rendering the tested cells unusable for further experiments.²⁰

Figure 2.1: Conventional barrier assessment methods. (A) TEER measurements in Transwell systems uses two electrode pair that are submerged in different compartments, measuring resistance of the cellular monolayer seeded onto the membrane. (B) Evaluating barrier by means of transport of fluorescently labeled dextran starts with cells cultured to a monolayer (B-I), then treated with a disease stimulus to change their permeability (B-II). After that FITC-dextran is added to the insert (B-III), and samples can be collected from the bottom compartment to measure the integrity of the cell layer (B-IV). Schematics of experimental setup for measuring hydraulic conductivity (C) starts with Transwell insert sealed in a chamber. For water transport, the reservoir is lowered to create a pressure gradient across the cell layer, then the water flux across the cell monolayer is measured using bubble tracker, and the hydraulic conductivity is determined accordingly (Reproduced with permission from *Annals of Biomedical Engineering* 38, 8 (2010). Copyright Springer Nature (2010)).

Quantification of paracellular diffusive transport of tracer molecules typically starts with cell seeding to a Transwell membrane (Fig.2.1B-I) followed by treatment of the cellular monolayer with a molecule of interest which would induce a change in permeability (Fig.2.1B-II). After treatment, a known concentration of a labeled tracer, such as FITC-

dextran, is added to the insert (Fig.2.1B-III) and over time, its diffusion over the cell layer is measured by taking repeated samples from the bottom well (Fig.2.1B-IV). The concentration of labeled dextran in individual samples can be calculated by measuring fluorescence intensity with a plate reader and standardizing against a calibration curve. If the increase in concentration is linear over time (which is typically only true in the initial stage of the experiment, when the concentration gradient between insert and well is still constant), the permeability coefficient of a solute can be calculated using Equation 2.3.

$$P = \frac{1}{C_i} \left(\frac{dC_w}{dt} \right)_0 \frac{V_w}{A} \quad (2.3)$$

Where the permeability coefficient P is a function of C_i , the initial concentration in the insert; $(dC_w/dt)_0$, the linear fit for the rate of increase in concentration at the start of the experiment; V_w , the volume of the well; and A , the culture area.

In order to isolate the permeability coefficient of the cell layer (P_{cell}) (Equation 2.4), a blank measurement in which permeability of the membrane (P_0) was established according to the method above, should be subtracted from the measured permeability of cells grown on the membrane (P_{total}).

$$\frac{1}{P_{cell}} = \frac{1}{P_{total}} - \frac{1}{P_0} \quad (2.4)$$

In addition to aforementioned techniques, measuring the flux of water across a cellular monolayer, also known as the hydraulic conductivity of a tissue, is another method to assess the barrier function of cultured cells. When performing measurements of hydraulic conductivity, water flux is facilitated by a defined pressure gradient. In addition, hydraulic conductivity can also be used to determine the optimal transmural pressure required to prevent delamination of endothelial cells from scaffold walls, which is a common challenge in micro vessel engineering.²⁶⁻²⁹ Hydraulic conductivity can vary similar to permeability across cellular monolayers found in different locations in the human body (e.g. endothelial cells). *In vitro* endothelial monolayers may be optimized to produce tighter or leakier vessels to water by using different cell material and exposing the cells to different shear stresses and pressures to model different tissues, such as the tight blood-brain barrier or the permeable kidney glomerulus.²⁹⁻³² *In vitro* measurements of hydraulic conductivity were demonstrated by Li *et al.* using a Transwell system (Fig.2.1C).³³ First, cells were grown on the Transwell insert to a monolayer. The filter was then sealed within a chamber. This chamber was connected to a water reservoir by a Tygon and borosilicate glass tube (Fig.2.1C). A difference in hydrostatic pressure was created across the filters by adjusting the height difference between the reservoir and the fluid covering the cell layers. Flow of water across the cell layers was then measured by tracking the position of a bubble pre-inserted into the glass tube. Using the volumetric flow rate derived from the displacement of the air bubble, hydraulic conductivity (L_p) can be calculated by (Equation 2.5);

$$L_p = \frac{J_v}{A \times \Delta p} \quad (2.5)$$

Where J_v is volumetric flow rate, A is the surface area of the Transwell insert and Δp is the hydraulic pressure difference across the cell layer.

2.2.2 Organ-on-a-Chip Systems

When using conventional *in vitro* systems barrier function of tissues is often found to be decreased compared to the physiological *in vivo* situation. For example, *in vivo* values of barrier tightness of the BBB have been reported to be larger than what can be achieved with simple *in vitro* systems.³⁴ Since inclusion of biochemical and mechanical stimuli that the cells would normally experience in their *in vivo* microenvironment has such an impact on their barrier function, there is a need for advanced conventional models that incorporate such factors. Therefore, in order to meet the shortcomings of the conventional models, microfluidic OOC systems have been developed. These systems provide a clearly defined, well-controlled physicochemical microenvironment for cell and tissue organization. Cells are exposed not only to fluid shear stresses by perfused microchannels, but also forces such as mechanical cyclic strain similar to what they would normally experience in living organs during processes such as breathing as in the case of lung-on-a-chip device reported by Huh *et al.*⁵ In another example, electric fields can be incorporated into these systems to pace contractile cells.³⁵ As a result, OOCs demonstrate functional realism that is normally not found in other *in vitro* systems. Despite their improved functional realism, OOCs devices typically require dedicated measurement setups and present specific challenges for assessing tissue barrier function. The following section provides an overview of how barrier function is typically assessed in OOC systems along with their unique challenges.

Figure 2.2: Integrated electrodes for measuring TEER in a BBB-Chip. (A) Exploded view of the device with top channel (TC), membrane (M), bottom channel (BC) and platinum wire electrodes (E1, E2, E3, E4). (B) Assembled device. (C) Schematic top view of the device. (D) Cross section schematic of the device showing endothelial cells (EC) cultured on membrane M in the TC. (E) Simplified equivalent circuit of the device, showing electrodes E1-E4, resistors representing the TC (R_1 and R_3), resistors representing the BC (R_2 and R_4) and resistor R_m representing the membrane and EC barrier. (Reproduced with permission from Biosensors and Bioelectronics 85 (2016). Copyright Elsevier)

Conventional TEER measurement setups (*i.e.* EVOM) are mostly confined to static and macroscopic cellular environments. Therefore, they are not suitable to be used in microfluidic systems due to the small scale of the devices which makes electrode placement in close proximity to the cells impossible. This leads to variations between measurements when electrodes are not firmly secured in the same positions. Integrating the electrodes directly into an OOC model and placing the electrodes closer to the cellular monolayer can reduce the influence of electrical resistance from the cell culture medium and the noise generated by any electrode motion. Moreover, electrodes can be scaled relative to the size of the microchannel dimensions within the system, thus compared to conventional systems, TEER can be measured with much smaller surface areas in OOCs. However, one needs to ensure a uniform current density across a cellular monolayer.²⁰

Figure 2.3: Typical OOC devices containing two adjacent channels separated by a semi-permeable membrane. (I) Device of Achyuta *et al.* that consists of 2 parts which are assembled following the cell seeding. (Reproduced with permission from Lab on a Chip 13,4 (2012). Copyright 2012 The Royal Society of Chemistry) (II) Device of Huh *et al.* that consists of two adjacent channels separated by a porous PDMS membrane. (Reproduced with permission from Science 328, 5986 (2010). Copyright 2010 American Association for the Advancement of Science) (III) Design of Kim *et al.* that contains two channels one of which seeded with gut epithelial cells, other containing the interstitial fluid. (Reproduced with permission from Lab on a Chip 12, 12 (2012). Copyright 2012 The Royal Society of Chemistry)

As mentioned before, OOCs can incorporate physiologically relevant fluid flow to study cells in conditions that resemble the *in vivo* situation more closely. Thus, TEER has been commonly used to evaluate functionality of several barriers including BBB, gastrointestinal tract and pulmonary tissues.^{5,6,36-47} An example of such a system is the BBB-chip reported by Van der Helm *et al.*, a multi-layered microfluidic device comprising two polydimethylsiloxane (PDMS) parts with defined microchannels, separated by a membrane made of polycarbonate, and containing 4 integrated platinum electrodes (200µm in diameter) that are inserted into the culture channels through side channels in the PDMS (Fig.2.2).^{44,48} For TEER measurements, a lock-in amplifier with a probe cable circuit is coupled with two of the four electrodes. A series of six resistance values is recorded using all the possible pairs of electrodes. Subsequently, Gaussian elimination is used to determine the resistance of the cellular barrier and membrane from these six resistance values. The four-electrode system enables direct isolation of barrier resistance regardless of variations in the system (*e.g.* temperature fluctuations and changes in medium solute concentrations)

affecting the inherent resistance of the system. Resulting TEER values obtained using this method were comparable to the values obtained by conventional Transwell systems.⁴⁴

Assessment of barrier quality of cells in OOCs with TEER has been challenging due to various factors. In these systems, temperature and physical support for cell culture as well as the characteristics of the electrodes such as material, quality and surface state have an influence on the TEER values.⁴⁰ Non-uniform current densities are a well-established source of measurement error for TEER in any system.^{40,49,50} but are particularly important in OOCs, due to the relatively low volume of medium in microfluidic channels resulting in high electrical resistance comparable to the cell layer resistance.^{40,49} In order to ensure a uniform current density and thus an equal potential drop over the entire cell culture area, one could integrate electrodes along the entire channel even though this might not be compatible with devices where mechanical deformations (*e.g.* stretching) are applied.⁴⁰ Alternatively, correction factors can be applied when calculating TEER from raw measurements to account for non-uniform current densities.^{40,49} Other potential sources of measurement errors are: chip-to-chip variation in positioning of the electrodes, air bubbles present in microchannels (different cross-sectional area). In addition to these physical sources of measurement errors, variations can be caused by incomplete cell coverage, even though cells of interest in the monolayer express cell-cell junction proteins. A slight gap (0.4%) in cell coverage can potentially reduce the TEER measured by 80%⁴⁰. It is essential to control all these sources of variation to enable comparison of TEER values between different microfluidic systems.

Assessment of a cellular barrier by means of resistance provides label-free, real time information, but it does require dedicated measurement setups as well as device designs. Therefore, assessment of barriers by measuring transport of tracer molecules is used more often in OOC systems. An example of such a system is the BBB-on-chip model of Achyuta *et al.*, which involves two cell types cultured on microchannels assembled into a chip (Fig.2.3-I). In this device, barrier integrity was measured using diffusion of fluorescently labeled dextran (3 kDa), which was perfused in the vascular channel and collected at the neural layer. The amount of diffused dextran was measured using a plate reader.⁵¹ Another example of using fluorescently labeled molecules to assess barrier integrity is the lung-on-a-chip device used by Huh *et al.* (Fig.2.3-II).⁵ This device is a three-layered sandwich where two adjacent channels were separated by a porous membrane. Added to the upper alveolar layer, FITC-conjugated albumin transport was measured by sampling liquid flowing through the lower channel. Similarly, Kim *et al.* demonstrated the diffusion of fluorescently labeled dextran added to the upper channel of a gut-on-chip device, by taking hourly samples from the bottom layer (Fig.2.3-III).⁶ Contrary to the traditional Transwell systems, these devices are dynamic. That means culture medium is often continuously perfused through the top and bottom channels, which makes it impossible to measure an increase in concentration in the collecting channel by repeated sampling. The concentration of tracer molecules in all repeatedly collected samples will typically be constant, and will only depend on the barrier tightness and the residence time - the time for molecules to accumulate in the fluid of the collecting compartment as it flows through the chip. The residence time is dictated by the volumetric flow rate and the volume of the collecting channel. Typically, due to continuous perfusion and the low volume of compartments, residence times are short, which means that the effective “sampling time” that can be used to estimate $(dC_w/dt)_0$ for equation 3 is also short. This may lead to low effective concentrations of tracers to reliably estimate the rate of increase. To overcome this issue,

Figure 2.4: Microfluidic system reported by Moya *et al.* for 3D vasculature modelling. (a) PDMS based microfluidic device contains outer microfluidic channels that connect to a series of central micro tissue chambers through a communication pore on each side. (b) central chamber is inhabited by endothelial cells and stromal cells embedded in a fibrin matrix (cross-section of panel (a) indicated by a black dotted line). (c) hydrostatic pressure is necessary for media flow and is enabled by large media reservoirs. (d-e) Microfluidic system enables robust interconnected vessel network formation within 14-21 days. (scale bar= 200 μm) (Reproduced with permission from *Tissue Engineering Part C: Methods* 19, 9 (2013). Copyright 2013 Mary Ann Liebert Inc.)

multiple measures can be taken: flow rates in the channels can be decreased, concentrations of tracer molecules in the source channel can be increased, and smaller tracer molecules with higher permeability coefficients can be used. Another complication of using this method in OOCs is that differences in pressures or hydraulic resistances between channels, although small, might cause transport of tracer molecules through advection instead of diffusion. Therefore, one should be careful with the fluid levels in different inlets to be equal to prevent any pressure differences.

OOCs are becoming progressively more 3D to mimic *in vivo* tissue structure and function. This means that many OOCs now contain 3D vessels or networks of vessels. Assessment of barrier function in such devices with 3D culture area geometries typically relies on imaging of fluorescent tracers, because measuring TEER is currently not possible due to challenges related to integration of electrodes as well as ensuring a uniform electric field along the culture area.⁵² An example of a device with a 3D vascular architecture is provided by Moya *et al.* They reported a microfluidic device with individual cell culture chambers self-assembled into a capillary network in the presence of cell media supplemented with growth factors (Fig.2.4).⁵³ Permeability of vessels could be assessed by injecting fluorescent dextran (70 kDa and 150 kDa) to the channels followed by imaging with fluorescence microscopy. Typically, this type of microscopy data is reported to make a qualitative or semi-quantitative statement about barrier function.

Still, microscopic tracking of tracer molecules can in principle be used to make quantitative statements about barrier function in OOCs with 3D geometries. For instance, Herland *et al.* constructed a 3D blood vessel-on-a-chip inside with lumens created by viscous finger patterning in a collagen I matrix.⁵² Cells seeded inside these lumens created a 3D vessel. Barrier quality in these systems was evaluated by infusing a fluorescently labeled dextran (3 kDa) followed by continuous recording of fluorescent images. Using these images, the apparent permeability coefficient (P_{app}) can be calculated by analyzing the total fluorescence intensity in an area and applying:

$$P_{app} = \frac{1}{\Delta I} \left(\frac{dI}{dt} \right)_0 \frac{r}{2} \quad (2.6)$$

Where ΔI is the step increase in the total fluorescence intensity upon adding dextran, $(dI/dt)_0$ is the initial rate of increase in intensity as dextran diffuses out of the vessels into the surrounding gel, and r is the radius of the tube.⁵⁴ For this type of measurements, it is essential to ensure a stable monolayer of cells in the beginning of the dye addition, otherwise if diffusion of fluorescently labeled molecules is too fast to reliably establish the intensity step ΔI , and quantification will not be possible.

2.3 Overview of Assessing Barrier Integrity in Organs-on-chips

Since their inception, a wide variety of OOC designs have been optimized to model various tissues of the human body. By the controlled incorporation of physiologically relevant forces, flows and geometries that are also found in their native *in vivo* environment, one can better recapitulate tissue and organ level physiology. Below is an overview of different OOC systems used to assess cellular barriers of different tissues. The list includes only a small fraction of a vast number of models as the focus of this chapter was restricted to models investigating cellular barriers until 2018 (Table 2.1).

Following abbreviations were used to describe each of the techniques: TEER for trans-endothelial/epithelial electrical resistance, PTT for paracellular transport of tracer molecules. Each row of the Table 2.1 has been classified based on the organ of interest, barrier assessment method (whether TEER, PTT or combination), co-culture (presence of multiple cell types in the same model), culturing type (whether cells have been cultured in hydrogels to provide a 3D microenvironment).

As it is challenging to setup TEER for 3D culture areas due to previously stated factors, it has not been preferred in many models, and instead barrier function is more often evaluated using PTT method.^{56,61} Moreover, it has been reported that the cells exposed to mechanical forces exhibit increased paracellular permeability even though TEER values for the cell layer remains stable, possibly due to increased transcytosis.⁶ On the other hand, the assessment of barrier function by TEER has unique advantages, as it can be performed continuously, non-invasively and in a controlled atmosphere. Moreover, technical proof-of-concept studies in impedance spectroscopy have demonstrated that also in 3D cell culture, electrical signals from integrated electrodes can still provide information about *e.g.* cell numbers and barrier properties in chips.^{73,74} Therefore, whenever possible, a combination of both methods should be applied to ensure a reliable readout on the barriers.

Table 2.1: Overview of Barrier Assessment Techniques in OOC Systems

Organ	Assessment	Co-culture	Culturing type	Reference
Blood- brain Barrier	TEER	No	2D	41,46
Blood- brain Barrier	PTT	No	2D	60, 55
Blood- brain Barrier	PTT	Yes	2D	56, 67
Blood- brain Barrier	PTT	Yes	3D	57,56
Blood- brain Barrier	PTT	No	3D	57
Blood- brain Barrier	TEER	Yes	2D	58
Blood- brain Barrier	TEER, PTT	Yes	2D	36,46,59,60
Blood- brain Barrier	TEER, PTT	Yes	3D	41,61,62
Cornea	PTT	No	2D	63
Gastrointestinal Tract	PTT	Yes	2D	64,65
Gastrointestinal Tract	TEER	Yes	2D	66
Gut	TEER, PTT	No	2D	6
Kidney	TEER, PTT	Yes	2D	38
Liver	PTT	No	2D	67
Lung	TEER, PTT	Yes	2D	5
Lung	PTT	Yes	2D	68
Retina	PTT	Yes	2D	69
Retina	PTT	Yes	3D	70
Vasculature	PTT	No	3D	53,71
Multiple organs	TEER	Yes	2D	72
Multiple organs	TEER, PTT	Yes	2D	46

2.4 Future Technical Development of Mimicking Barriers in Organs-on-chips

As is clear from Table 2.1, many different barrier tissues have already been modeled with OOC technology. As OOC technology is developed further, the 3D culture configurations will become increasingly complex. This can already be observed in recent studies that focus on advanced 3D scaffolds for alveolus-on-chip⁷⁵ and colon-on-chip systems.⁷⁶ Obviously,

this increasing complexity will also lead to challenges in measuring barrier function, *e.g.* with interpreting signals from electrical sensors. It will therefore be important to keep developing innovative read-outs and more sophisticated sensor technology, such as 3D biocompatible electrodes that can directly integrate in the cultured tissues.⁷⁷ Most OOCs are currently fabricated from PDMS, but the high gas permeability of this material makes it challenging to control specific gas pressures in an OOC system. Local control over gas concentrations are important in studying the transport of gases over barrier tissues in for example lung-on-a-chip or the vessel-on-a-chip systems. In addition, the barrier function of many tissues is affected by local oxygen concentrations. For example, the permeability of blood vessels changes dynamically in episodes of ischemia and reperfusion⁷⁸ and the barrier function of intestinal epithelium is affected by interactions with anaerobic bacteria that only survive in low-oxygen conditions⁷⁹. Another challenge when using PDMS-based devices is selective adsorption and absorption of molecules from the culture media, which in turn reduces their effective concentrations and ability to affect cells. This is especially important in drug efficacy or toxicology studies where compound availability to the cells is required to determine the dosage and efficiency of the drug⁸⁰. To reduce absorption, PDMS channel surfaces are often coated to block the passage of compounds.⁸¹ Alternatively, OOC systems are increasingly being manufactured with materials other than PDMS, such as polystyrene, glass, and cyclic olefin copolymer to allow control over gas pressures as well as to prevent absorption of compounds.^{82,83}

Combined with the engineering of OOC systems that contain ever more physiologically relevant cues, *in vitro* barrier models will also integrate progressively more cell types. By doing so it will be possible to mimic cellular dynamics as well as crosstalk between cell types, such as barrier tissue cells and immune cells. However, one needs to be cautious about the media compatibility when incorporating several cell types. Cells may not survive in each other's respective medium and continuous fluid flow may be needed to allow locally stable culture conditions for various cell types. Together with an increasing 3D complexity of OOC systems, this will require innovative solutions for microfluidic actuation, for example by 3D printing parts of OOC systems.

2.5 Conclusion

Barriers exist in our bodies to maintain homeostasis and protect vital organs. Disruption of tissue barriers lead to various diseases. It is undeniable that investigation of these barriers in diseases might reveal new mechanisms and treatments. Therefore, proper *in vitro* tools are required to evaluate the integrity and characteristics of barriers. Conventional Transwell systems suffer from not fully recapitulating the complexity of the microenvironment as well as inclusion of physical forces that have an impact on the development and differentiation of cells. Microfluidic OOC systems are great tools to overcome these challenges. In addition to integrating physical stimuli, they consist of additional co-cultures with immune cells and microbes to mimic the physiological tissue environment more realistically, thus giving more accurate information about underlying organ physiology and disease mechanisms.⁸ Due to their complexity and a wide plethora of designs, it can be challenging to compare measurements performed in a specific OOC system with data from other OOCs or conventional assays. To overcome this challenge, it is essential to implement assays that quantitatively measure barrier function, independent of exact system design. Assessing barrier function will be of importance especially in cases where multiple organ models are

combined to create body-on-chip systems, where barrier functions influenced by other organ models (*e.g. via* inflammation) can be studied. Incorporating such measurements in OOCs may require adjustments or corrections to avoid common measurement errors, as discussed in this review.

Finally, advances in stem cell technology and access to patient-derived cells will improve physiological relevance of current OOC models and will contribute to more realistic and patient-specific disease models. Continuous monitoring of barriers without disrupting viability of the cells in such OOC models will yield unique insights in mechanisms of disease, thus contributing to the development of patient-specific treatments in the context of precision medicine.

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3

Microfluidic Organ-on-a-Chip Model of the Outer Blood-Retinal Barrier with Clinically Relevant Read-Outs for Tissue Permeability and Vascular Structure

The outer blood–retinal barrier (oBRB) tightly controls the transport processes between the neural tissue of the retina and the underlying blood vessel network. The barrier is formed by the retinal pigment epithelium (RPE), its basal membrane and the underlying choroidal capillary bed. Realistic 3D cell culture based models of the oBRB are needed to study mechanisms and potential treatments of visual disorders such as age-related macular degeneration that result from dysfunction of the barrier tissue. Ideally, such models should also include clinically relevant read-outs to enable translation of experimental findings in the context of pathophysiology. Here, we report a microfluidic organ-on-a-chip (OOC) model of the oBRB that contains a monolayer of human immortalized RPE and a microvessel of human endothelial cells, separated by a semi-permeable membrane. Confluent monolayers of both cell types were confirmed by fluorescence microscopy. The 3D vascular structures within the chip were imaged by optical coherence tomography: a medical imaging technique, which is routinely applied in ophthalmology. Differences in diameters and vessel density could be readily detected. Upon inducing oxidative stress by treating with hydrogen peroxide (H_2O_2), a dose dependent increase in barrier permeability was observed by using a dynamic assay for fluorescence tracing, analogous to the clinically used fluorescence angiography. This OOC of the oBRB will allow future studies of complex disease mechanisms and treatments for visual disorders using clinically relevant endpoints *in vitro*.

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3.1 Introduction

Vision loss and blindness are estimated to affect approximately 314 million people globally.¹ Visual impairment dramatically affects quality of life for patients, and causes major direct and indirect costs related to healthcare. In order to study disease mechanisms and to develop new treatment strategies, experimental models that realistically mimic tissues in the human eye are essential. The outer blood-retinal barrier (oBRB) is one of the key ocular structures for which the development of new model systems is needed, as it is involved in the pathophysiology of various visual disorders, of which age-related macular degeneration (AMD) is the one with the highest prevalence. AMD is a progressive chronic disease that affects vision in nearly 9% of the worldwide population. This number is expected to increase even further as the global population ages in the coming decades.²

AMD is a result of dysfunction of the key tissues in the oBRB: the retinal pigment epithelium (RPE), the underlying collagenous membrane, known as ‘Bruch’s membrane’ and the adjacent choroidal capillary bed. There are two types of AMD: the “dry” and “wet” forms. Dry AMD is a chronic disease that can progress into severe vision loss. It is characterized by accumulation of insoluble, extracellular aggregates of proteins and lipids in the retina, called “drusen”. As the disease progresses to a late stage called “geographic atrophy”, there is a considerable loss of RPE cells as well as overlying photoreceptors, which rely on the RPE for nourishment and waste disposal.³ In contrast, “wet” AMD only corresponds to 15% of the cases of AMD but is responsible for the majority of cases of AMD-related vision loss.⁴ This form is characterized by choroidal neovascularization (CNV), in which new blood vessels arise and breach the normal tissue barriers of the outer retina from the underlying choroid. These new vessels also leak fluid below or within the retina, which can cause sudden loss of central vision.

Environmental and genetic factors are involved in the pathogenesis of AMD. Non-genetic risk factors include cigarette smoking, older age and obesity.⁵ Furthermore, genetic studies have identified associations of several important biological pathways with AMD pathology: the complement system, extracellular matrix remodeling, lipid metabolism, and angiogenesis signaling pathways.^{6,7}

Oxidative stress due to accumulation of reactive oxygen species (ROS) is a key factor in the pathophysiology of AMD.^{8,9} Patient retinas have increased local accumulation of lipofuscin which generates ROS,¹⁰ they have mitochondrial DNA damage due to ROS,¹¹ and there is a marked increase in glycation end-products and peroxidized lipids.¹² As this oxidative stress due to ROS production is toxic to the cells, this might lead to increased permeability of the choroidal blood vessels (*e.g.* leakage), which is a hallmark of wet AMD.

From a clinical perspective, it is important to closely monitor the disease progression of AMD in a patient, for example to detect when the disease progresses from dry AMD, for which there are no treatments, to wet AMD, which can be treated with intraocular injections of anti-angiogenic drugs. The clinical assessment is performed using medical imaging modalities, particularly optical coherence tomography (OCT) and fluorescence-based angiography (FA).^{13,14} OCT is used to reconstruct high-resolution 2D or 3D images that visualize (abnormalities in) the individual retinal cell layers and blood vessels of the choroid. It is based on the measurement of ‘light echoes’ as a function of tissue depth, through the interference of a reference beam with the light that has been backscattered from

the retina.¹⁵ FA relies on the perfusion, clearance and leakage of intravenously injected hydrophilic fluorescent tracers.¹³ As normally these dyes do not cross the blood-retinal barrier, any defect that compromises the barrier integrity will result in abnormalities on FA.¹⁶

To better understand the pathophysiology of AMD, experimental models are required in which the morphological changes of the tissues can easily be observed, and the experimental conditions can be readily manipulated. Rodent models are disadvantageous due to the lack of a macula and interspecies anatomical differences. Non-human primates are more realistic models, but the time required for the disease progression, the high costs and the ethical issues make primate models less suitable for fundamental biomedical research.¹⁷

Due to the recent developments in human pluripotent stem cell technology, *in vitro* models of the oBRB are becoming increasingly sophisticated. However, most of these models still rely on simple monolayers of cells (typically monocultures of human pluripotent stem cell-derived RPE) on plastic surfaces or membranes, and therefore these models do not fully recapitulate the 3D and tissue-level physiology.¹⁸

In recent years, OOC devices have proven to be promising disease models.¹⁹⁻²⁶ OOCs are microfluidic cell culture devices with engineered microchannels that are continuously perfused and inhabited by living cells to form tissues that exhibit organ-level physiology. Depending on the research question, complexity of these systems can be adjusted by systematically including cell types and physico-chemical parameters of the tissue microenvironment in a well-controlled manner. The simplest systems consist of a single perfused microfluidic chamber and one type of cultured cell, whereas more complex devices have two or more microchannels that are separated by porous membranes lined by two or more cell types, which then simulate the interface between different cell types.²² Mimicry of tissue complexity can be further increased by the inclusion of 3D structures, for example by including organoids or hydrogels based on extracellular matrix proteins.²⁷⁻²⁹

Recently, multiple OOC models of the outer layers of the human retina have been reported.³⁰ One of the models relies on 2D co-cultures of RPE and endothelial monolayers in microfluidic chips.³¹ One reported model also includes co-cultured retinal organoids in addition to a monolayer of RPE.³² The other two models consist of co-cultures of RPE and self-developed 3D capillary endothelial networks.^{33, 34} Due to their 3D nature, these latter models allow studies of on-chip neovascularization in response to hypoxia and growth factor stimulation, thereby clearly demonstrating the potential of applying OOC models of the oBRB in studies of AMD. However, because these models rely on endothelial cell self-organization into microvascular networks, researcher's control over 3D vessel geometries is limited. More control over vessel geometry would strongly improve the reproducibility of these models.

In terms of read-outs, some of the current OOC models do include tracking of fluorescent tracers in order to assess the cell coverage by comparison of cell-containing channels with empty devices.³⁵ In contrast, none of the current OOC models of the oBRB use OCT as an additional read-out. Previously, OCT has been used in OOCs as a functional tool to monitor and characterize cell specific functions, demonstrating the feasibility of using this imaging modality as a read-out.³⁶ The inclusion of clinically relevant read-outs will be especially important for validation of devices and to facilitate the transfer of knowledge between researchers and ophthalmologists.

In the present study, we report an OOC model of the oBRB, based on co-cultures of RPE and human umbilical vein endothelial cells (HUVECs) in a microfluidic chip that contains a microchannel and an open-top culture chamber separated by a polyester membrane. In the microchannel, we created a microvessel with a well-defined geometry within a collagen I hydrogel using a subtractive method of micropatterning.³⁷ We used fluorescent tracers to track permeability of the microvessels and the RPE. Moreover, we implemented OCT as an innovative and clinically relevant read-out for our OOC model and used it to confirm sizes and structural changes of the engineered microvessels. Using this model, in contrast to existing models of AMD, we investigated one of the early hallmarks of wet AMD: the increased permeability of blood vessels due to ROS exposure.

Figure 3.1 PDMS-based OOC device allows 3D co-cultures of monolayers of human RPE cells with human endothelial cells lining a microvessel. (A) The device contains (i) a bottom compartment with a defined ($1\text{ mm} \times 1\text{ mm}$) microchannel, (ii) a polyester membrane with $8\text{ }\mu\text{m}$ pore size ($\sim 10\text{ }\mu\text{m}$ thickness), (iii) a middle compartment which contains an open-top culture chamber (3 mm in diameter) and 2 inlets (1.2 mm in diameter) and (iv) a top compartment that consists of three reservoirs for media storage (5 mm in diameter). (B) Schematic representation of patterning collagen I hydrogel using the subtractive method of patterning. This process starts with the hydrogel being pipetted into the channels from side inlets. This was followed by the insertion of two blunt needles (outer diameter of 0.9 mm) each inserted from side inlets and a syringe needle (outer diameter of 0.5 mm) inserted through the needles along the channel. After gelation of the collagen inside the microchannel, needles were removed to reveal the microvessel structure. For illustrative purposes, reservoir layer was removed. (C) Immunolabelling of the cells revealed a continuous distribution of each cell type. (i) 3D construction of confocal microscopy image of the co-culture area stained for nuclei and actin filaments indicates a monolayer alongside the channel. (ii) Top surface of the microvessel shows an equal distribution of HUVECs along the microchannel. (iii) Cross-sectional microscopy image of the co-culture area as indicated by DAPI (nuclei) and actin filaments staining reveals the circular structure of the microvessel occupied by HUVECs, as well as ARPE-19 cells that are located above the microvessel. These cells were positive for their respective cell-cell adhesion markers; (iv) VE-Cadherin expression for HUVEC, (v) ZO-1 expression for ARPE-19. Scale bars: $50\text{ }\mu\text{m}$.

3.2 Materials and Methods

3.2.1 Chip Fabrication

Poly (methyl methacrylate) (PMMA) (Altuglass) master molds of the channel structures were designed with SolidWorks and fabricated with a computer numerical control (CNC) milling machine (Datron Neo, Datron AG). PDMS base and curing agent were mixed in a 10:1 wt ratio (Sylgard 184 Silicone elastomer kit, Dow corning). After degassing the mixture, it was poured onto the fabricated positive PMMA molds and cured for at least 3h at 60 °C. Following curing, PDMS was removed from each one of the molds as slabs. PDMS slabs with microchannel imprints ($1 \times 1 \text{ mm}^2$) were cut from each side to generate side inlets for the insertion of needles for microvessel patterning. After that, using biopsy punches (Robbins Instruments) 2 inlets and 1 culture chamber (1.2mm and 3mm in diameter respectively) were punched into the PDMS slab which corresponds to the middle part of the final assembled device (Fig.3.1A-iii). In addition, 3 reservoirs were punched (5mm in diameter) into another PDMS slab for the top compartment of the assembled device (Fig.3.1A-iv). After that, all three slabs were aligned and cut into device-sized pieces. Before assembly of the parts, dust was removed using Scotch tape (3M). Leakage-free bonding of the parts with a membrane (Fig.3.1A-ii) in between them was achieved by using a PDMS/toluene mortar (5:3 wt ratio) (toluene from Merck) as reported previously.^{38, 39} This mixture was spin-coated onto a glass coverslip (1500rpm, 60s, 1000rpm/s, Spin150, Polos) and transferred to the device parts using an ink roller (Fig. 3.1A-i,iii). After that, a $\sim 36 \text{ mm}^2$ piece of polyester membrane with a pore size of $8 \mu\text{m}$ (GVS Life Sciences) was aligned and sandwiched between the center of the bottom (Fig.3.1A-i) and middle (Fig.3.1A-iii) compartments. This was followed by overnight baking the parts at 60°C. Final assembly of the device was done by exposing the surfaces of top compartment (Fig.3.1A-iv) and the pre-assembled device (Fig.3.1A-i-iii) to air plasma (50W) for 40s (Cute, Femto Science). After plasma treatment, activated surfaces were pressed together.

3.2.2 Surface Functionalization of PDMS

Internal surfaces of PDMS devices (microchannels and culture chambers) were functionalized using (3-aminopropyl) triethoxysilane (APTES, Sigma Aldrich) and glutaraldehyde (Sigma Aldrich). Following the aforementioned assembly of the final device with plasma activation of surfaces, 3% (v/v) APTES mixed in ultrapure H_2O (ELGA) was added into the channel and reservoir, and incubated at room temperature (RT) for 5min. Following APTES coating, the chips were rinsed thoroughly with 100% ethanol and incubated for 5min in 100% ethanol to eliminate the remaining APTES. After air-drying the devices, 10% glutaraldehyde was added to the channel and the culture chamber, and the devices were incubated for 5min at RT. This was followed by thorough rinsing with distilled H_2O and drying for 2h at 60°C prior to collagen patterning.

3.2.3 Hydrogel Patterning

Collagen Type I Gel Patterning: Before the addition of collagen, inlet and outlet holes were sealed with scotch tape (3M) on top of the media reservoirs to prevent collagen from filling these reservoirs. The devices were kept on ice for at least 30min before patterning in order to avoid premature and incomplete collagen gelation in the channels. Rat tail collagen type I (VWR) was prepared according to the manufacturer's instructions at a concentration

of 6mg/ml and a pH between 7.5 and 8 by mixing with dH₂O, phosphate buffered saline (PBS) and 1N sodium hydroxide solution. Collagen solution was then pipetted into the channel through one side of microchannel (Fig.3.1B-hydrogel addition). This was followed by insertion of stripped blunt needles of different inner diameters (20G, 23G, 30G; METCAL) into the side inlets until underneath the inlet holes, as well as needles with various outer diameters (500 μ m (SurGuard2, VWR), 300 μ m and 120 μ m (J-type acupuncture needle, Seirin) with the cap removed guided through the blunt needles which have relevant inner diameters respectively (Fig.3.1B-needle insertion). This was followed by incubation of the devices at 37°C for 60min for collagen to gelate. Afterwards, the needles were drawn out of the side inlets to reveal the microvessel structures (Fig.3.1B-needle removal). After optical confirmation of successful patterning, the channels were sealed by inserting square polystyrene plugs (1mm², Evergreen Scale Models) into the side inlets. The plugs remained in place for all subsequent experiments. Finally, channels were flushed once more with culture media through the inlet holes and the devices were incubated overnight at 37°C, 5% CO₂ to normalize the pH prior to cell seeding.

Fibrin Gel Patterning: For OCT experiments, fibrin hydrogel mixed with HUVECs and fibroblasts was patterned to induce vascular network formation. Prior to patterning, devices were sealed with scotch tape as mentioned before, and remained on ice. This hydrogel solution was prepared using final concentrations of 3mg/ml fibrinogen (Sigma), 4U/ml thrombin (Sigma), 8 $\cdot$ 10⁶cells/ml HUVECs (Lonza), 1 $\cdot$ 10⁶cells/ml human lung fibroblasts (Lonza). These were mixed before pipetting the hydrogel into each channel via side inlets. Afterwards, scotch tape was removed and devices were incubated for 10min at 37°C, 5% CO₂ for fibrinogen to cure. Following curing, needles were removed and side inlets were sealed as mentioned before. The devices were refreshed using endothelial cell growth medium (ECGM-2: Basal medium (ECBM-2) with supplement mix (PromoCell GmbH) and 50U/ml penicillin/streptomycin (P/S, ThermoFisher) twice daily. Networks started to form after 5days of culture and stabilized on day 7. On day 7 cells were fixated using 4% formaldehyde for 15min at RT until OCT measurements.

3.2.4 Cell Culture and Seeding in Chips

Fibroblasts (Lonza), HUVECs (Lonza) and ARPE-19 (ATCC) cells were cultured with fibroblast growth media (FGM-3: Basal medium with supplement mix from PromoCell GmbH and 50U/ml P/S (ThermoFisher), ECGM-2 and DMEM/F12 (with GlutaMAX, ThermoFisher) supplemented with 10% fetal bovine serum (FBS; ThermoFisher) and 50U/ml P/S), respectively. HUVECs were cultured in T75 culture flasks coated with 0.1mg/ml collagen I (Rat tail collagen I, ThermoFisher) in PBS (ThermoFisher), whereas ARPE-19 cells and fibroblasts were cultured in non-coated T75 flasks. The cells were incubated at 37°C in humidified air with 5%CO₂. Flasks with confluent monolayers were either used for experiments or subcultured. HUVECs and ARPE-19 were kept in culture up to passage number 6 and 30, respectively. Prior to cell seeding, the culture chamber was rinsed with PBS and coated with 0.1mg/ml collagen I for 30min at 37°C. After that, the chamber was washed with cell medium to remove non-bound collagen. This was followed by obtaining HUVECs from a confluent flask using 0.05% Trypsin-EDTA (ThermoFisher). Suspended in fresh ECGM-2 media at 4.5 $\cdot$ 10⁶cells/ml, cell suspension was pipetted into the channel via the inlet. After optically confirming the cell suspension in the channels, the devices were placed upside down for gravity-driven seeding of the top of the lumens and incubated at 37°C and 5%CO₂ for 60min. After that, devices were taken out of the incubator,

and non-bound cells were removed by flushing the channels with fresh cell media. Next, to seed the bottom of the lumens and the culture chamber, HUVECs and ARPE-19 were freshly obtained from confluent flasks using 0.05% Trypsin-EDTA. After that, suspended in ECGM-2 at a concentration of $5 \cdot 10^5$ cells/ml for ARPE-19 and $4.5 \cdot 10^6$ cells/ml for HUVECs, cells were pipetted into the culture chamber and microvessel, respectively. After 60min of static incubation at 37°C and 5% CO₂, non-attached cells were washed away with fresh media, and reservoirs were filled with ECGM-2 medium to prevent drying. For media refreshing, a filtered pipette tip full of media was placed on the inlet, whereas an empty one placed in the outlet. Furthermore, the culture chamber was filled with media and sealed with scotch tape. The devices were then placed on a custom-built rocking platform inside a 37°C and 5% CO₂ humidified incubator. The rocking platform tilted back and forth at an angle of 60° at an interval of 45s to facilitate a gravity driven medium flow in channels. Media was replaced with fresh media daily.

3.2.5 Cell Staining

On-chip co-cultures were stained for actin filaments and nuclei for confirmation of cell monolayers on microvessel walls and inside the culture chamber. For that, devices were first washed with PBS and fixed with 4% formaldehyde (in PBS, ThermoFisher) for 15min at RT. The fixative was washed away with PBS and the cells were incubated in permeabilization buffer (PB), which contains 0.1% Triton X-100 (Sigma Aldrich) and 10mg/ml bovine serum albumin (Sigma Aldrich) in PBS for 60min at RT. Afterwards, the cells were incubated for 2h at RT with 12.5µg/ml 4', 6-Diamino-2-Phenylindole (DAPI, ThermoFisher) and 4drops/ml ActinGreen (binds to actin filaments, ThermoFisher) in PB. Following incubation, the cells were rinsed three times with PBS and then washed three times with PBS for 10min at RT.

For assessment of cell morphology under the exposure of H₂O₂, HUVECs on culture well plates were fixed for 10min at RT with 4% formaldehyde, followed by permeabilization in 0.1% Triton X-100 at RT for 15min. Afterwards, cells were stained by incubating with 1.25µg/ml DAPI and 3.75U/ml Alexa Fluor 633 Phalloidin in PBS for 1h at RT. After each step cells were washed 3 times with PBS.

As a confirmation of expression of specific adhesion markers, each cell type was stained on a glass cover slip coated with collagen I. Cells were seeded at a density of $5 \cdot 10^4$ cells/cm² and kept in culture conditions for a day to grow to a monolayer. This was followed by washing with PBS and fixing with 4% formaldehyde for 10min at RT. The fixative was washed away with PBS and the cells were incubated in PB for 15min at RT. Afterwards, HUVECs and ARPE-19 were incubated with goat anti-human VE-Cadherin IgG (1µg/ml in PB, R&D Systems) and mouse anti-human ZO-1 IgG (5µg/ml in PB, BD Transduction Laboratories) for 2h at RT, respectively. Following incubation, the cells were rinsed three times with PBS and then washed three times with PBS for 10min at RT. After that, the cells were incubated for 1h at RT with donkey anti-goat IgG Alexa Fluor 546 (2.5µg/ml, ThermoFisher) and chicken anti-rabbit IgG Alexa Fluor 488 (2.5µg/ml, ThermoFisher) in PB, respectively. After staining, cells were washed again with PBS. Afterwards, the coverslips were transferred to microscope slides for imaging.

The cells were imaged with phase contrast, fluorescence microscopy using the EVOS FL Cell Imaging System (Life Technologies; RFP filter (ex 531/40, em 593/40) for VE-Cadherin, GFP filter (ex 470/22, em 510/42) for ZO-1, Cy5 filter (ex 628/40, em 692/40)

for Phalloidin and DAPI filter (ex 357/44, em 447/60) for DAPI) and Nikon Confocal A1 Microscope (Nikon). Z-stacks, 3D reconstruction and Z-projections were all analyzed and reconstructed with Fiji software ⁴⁰.

3.2.6 Tracking Permeability in Co-Cultures On-Chip

The permeability of cell layers in the OOC co-cultures was visualized by a method analogous to the clinically used FA. Devices were cultured for 72h prior to experimentation. Afterwards, devices were first treated with ECBM-2 (with 2% FBS and 1% P/S) overnight. The following day, HUVECs were either left untreated or were exposed to 800 μ M and 10mM H₂O₂ dissolved in ECBM-2 (with 2% FBS and 1% P/S) for 1 day. Culture medium in devices was refreshed twice daily using the rocking platform with freshly made H₂O₂ solutions and ECBM-2. After 1 day of exposure, first, each device was mounted on EVOS FL Cell Imaging System (Life Technologies) with a 4X air objective. Then the bottom half of a 200 μ l pipette tip, acting as a reservoir, was fitted on the inlet. After that, a syringe pump was connected to the device via Tygon tubing (1mm inner diameter, Saint-Gobain Performance Plastics) which was placed on the outlet. After that, the reservoir was filled with 100 μ l of 60 μ g/ml 40kDa FITC-Dextran diluted in ECBM-2. This was followed by withdrawal of the dextran along the microchannel 25 μ l/min using a syringe pump (PHD ULTRA, Harvard apparatus). After ~4min of FITC-Dextran perfusion, the reservoir was emptied and replaced with 100 μ l ECBM-2 with which the channels were perfused (25 μ l/min). During the whole procedure, sequential images were taken with 10 s intervals for 9min. Using these images, the barrier formed by HUVECs was evaluated. First, intensities of a 20 by 20 pixel area of the gel (Fig.3.2B-top between white and red lines) and microvessel (Fig.3.2B-top between red lines) were corrected for background intensity by subtracting the average intensity of a 20 by 20 pixel background area (Fig.3.2B-top outside of white lines). Second, intensity of the gel was normalized to intensity of the microvessel. Using the fluorescence profile of normalized gel over time, the slope of the increase in intensity during the perfusion phase was calculated by linear fitting (MS Excel). This normalized slope was used as a measure for permeability.

3.2.7 Optical Coherence Tomography Measurements

OCT images were acquired with a home-built visible light system (Suppl.Fig.7.1.2) previously described ^{41, 42}. Briefly, the setup was based on an open-air Michelson interferometer, with a supercontinuum light source (SuperK Extreme EXB-6, NKT Photonics). A beam splitter (BS028, Thorlabs) guided 10% and 90% of the light towards the sample and reference arm respectively. Achromatic lenses (AC127-025-A, Thorlabs) with a focal length of 25mm focused the light on the sample and on a piezo-driven reference mirror. Those lenses determine the lateral resolution of the system, in this case 2.5 μ m in air. After interaction of light with the sample and the reference mirror, backscattered light was guided through a single mode fiber (S405-XP, Thorlabs) into a custom-built spectrometer. The spectrometer dispersed light onto a line-scan camera (Sprint spL4096-140km, Basler), achieving a spectral resolution $\delta\lambda=0.1$ nm over a range of 440-600nm (Suppl.Fig.7.1.2A). The spectrum acquired by the camera can be related to depth after processing, producing depth-intensity profiles known as A-scans. The system parameters described above led to a theoretical axial resolution of 1.4 μ m in air and an imaging depth of $\sim\pm 0.6$ mm. Chips were mounted on a motorized stage (T-LS13M, Zaber), controlled by a custom-built LabView algorithm in order to allow lateral scanning. Cross-sectional

Figure 3.2 Assessment of barrier integrity by ‘fluorescein angiography’ in devices. (A) Device schematics illustrating the location used in acquisition. (B) Illustration of the fluorescein angiography procedure (top) and sequential top-view images taken during the experimentation (bottom). Each column represents different stages of the procedure: before the dye perfusion (‘Background’), during the perfusion of the dye (‘Perfusion’), during the media perfusion (‘Removal’). As the dye was perfused through the microvessel (red dashed lines), diffusion occurs into the gel depending on the integrity of the barrier. Untreated (UT) is a positive control with HUVECs and ARPE-19 cultured in the microvessel and the culture chamber respectively. The ‘800 μ M’ and ‘10 mM’ rows represent the treatment concentrations of HUVECs with H₂O₂ for 5 days. Contrast in raw images were enhanced for illustrative purposes using a false-color lookup table (bottom). Scale bar: 200 μ m (C) A typical fluorescence intensity profile during FA is summarized using ‘untreated’ condition as an example, with different phases labeled in their respective colors, matched to the phases in panel B. The slope in the ‘Perfusion’ phase (‘ α ’) is used as a measure of permeability. (D) Normalized to the intensity in the microvessel, slope of the increasing gel intensity in the ‘Perfusion’ phase is summarized as a bar graph. Compared to untreated cells (UT), a significant increase (UT vs condition, Student’s *t*-test, $p < 0.05$) in permeability (denoted by an asterisk) was detected in longer exposures of higher H₂O₂ concentrations. Error bars represent standard error of the mean, all experiments were performed at least 6 times.

images (B-scans) were obtained by concatenating 350 A-scans spaced at 3 μ m, leading to a lateral scan range of 1.05mm. At every lateral position, 100 spectra were acquired with an exposure time of 800 μ s. The piezo-driven reference mirror allowed full extension of the imaging depth to $\sim$ 1.2mm, by removal of the DC-component and mirror-image in each A-scan. This was achieved by applying a procedure similar to our previous zero-delay acquisition.^{42, 43} In short, the movement of the piezo induces a frequency modulation as a function of time for each A-scan. By means of a band-pass filter in the frequency domain, the DC component and mirror image can be removed. Note that unlike our method for zero-delay acquisition, here the position of the reference mirror was kept constant, and the depth-axis was calculated from the spectrum. Finally, in order to mitigate the loss in resolution caused by dispersion of the PDMS layers in the chip, a flat layer of PDMS with the same thickness of the chip was introduced in the optical path of the reference beam.

Microvessel quality was evaluated with a custom-built Matlab ⁴⁴ script. First, OCT images were recorded at 4 different cross-sections alongside the microchannels containing various sized microvessels (n=32 for each size). Second, images were filtered using a Gaussian convolution to reduce speckle and then binarized. Third, a rough region of interest (ROI) was drawn on the microvessel. The extremes of the microvessel were detected and their respective locations marked as coordinates, physical coordinates were calculated assuming a refractive index of 1.4 as for PDMS. Using these locations, a circular shape was drawn around the central point of the four marks with theoretical diameters of 500, 300 or 120µm. Fourth, the contents of the ROI were analyzed by comparing the features in the image to the desired shape of the microvessel. Finally, we defined a quality factor Q_{vessel} , where 1 indicates perfectly circularly microvessels. The quality of the microvessel (Q_{vessel}) was calculated as ratios between areas inside and outside the circular ROIs, as follows,

$$r_{inside} = \frac{A_{inside}}{A_{circle}} \quad (3.1)$$

$$r_{outside} = \frac{A_{outside}}{A_{circle}} \quad (3.2)$$

$$Q_{vessel} = \frac{r_{inside}^2}{(1+r_{outside})^2} \quad (3.3)$$

where A_{inside} and $A_{outside}$ are the areas of features inside and outside the theoretical circle respectively, as well as A_{circle} which is the area of the circle itself. A minimum threshold of 0.5 was set for selecting proper microvessels.

Diameters of the branching vessels within patterned fibrin hydrogel were calculated by fitting circles into each observed cavity and measuring their diameter using a custom-built Matlab script ⁴⁴.

3.3 Results and Discussion

3.3.1 Co-culture of retinal pigment epithelium and vascular endothelium in the microfluidic chip

In the present study we aim to mimic the organization of the outer lining of the retina in an OOC model. The chips were designed to have two compartments separated by a porous membrane (Fig.3.1A), resembling the layered structure of the RPE and the underlying choroid in the human retina. After the patterning of microvessels, HUVECs and ARPE-19 were introduced in the device. After 72h of culture, confocal microscopy inspection of the co-cultures stained for actin cytoskeleton and nuclei revealed a homogeneous distribution of the cells, which suggests that there were continuous cell layers (Fig.3.1C). To confirm each of these specific cell types, ARPE-19 and HUVECs were fluorescently stained for the specific cell-cell adhesion molecules ZO-1 and VE-Cadherin, respectively (Fig.1C). Subcellular localizations were as expected, with localization of the respective proteins at cellular junctions.

We chose to set up our model with ARPE-19 and HUVEC, because they have both been used extensively in modelling the oBRB *in vitro*. ⁴⁵⁻⁴⁷ The purpose of our current study was to establish relevant read-outs, and we therefore chose well-characterized cells as a point of reference. Still, it is clear that ARPE-19 has only limited relevance when modelling the

Figure 3.3 Imaging matrix structure and microvessels in the OOC system using OCT. (A) Schematic overview of the OOC device with inset showing the cross-section view of the channel. (B) Measured diameters of collagen I hydrogel patterned microvessels with different needle dimensions. Horizontal (Hor, x-axis in (A)) and vertical (Vert, y-axis in (A)) are measured separately. Each dot represents a different cross-section along the channel. Line showing mean with error bars denoting standard error of mean. Theoretical distances were labelled as dotted lines at 500, 300, and 120 μm . (C- i) Distribution of quality assessment of microvessels with different diameters. Values below 0.5 were considered as improper. (C- ii- iii) OCT images of two different vessels (500 μm) with qualities at opposite extremities were shown with their respective quality factors. (D) OCT cross section of a microvessel containing collagen I patterned hydrogel without cells (D- i) and fibrin, HUVECs and fibroblasts with white arrows showing cavities representing branches within the hydrogel (D-ii). Blue arrows represent OCT imaging artefacts. Scale bars: 100 μm . (D- iii) Distribution of vessel sizes within the fibrin hydrogel based on image analysis of multiple OCT cross-sections.

RPE, as it lacks pigmentation and other hallmarks of RPE *in vivo*. Similarly, HUVECs are a popular source of primary human endothelial cells, but its relevance for modelling the choroid is limited to cell type. Modelling the choroid is challenging in any case, because even primary human choroidal endothelium loses choroidal endothelial characteristics, *e.g.* vascular markers and fenestrations, when cells are isolated and expanded in culture.^{48, 49} Undoubtedly, stem cell-derived choroidal endothelial and RPE cells are what is ultimately needed to create an OOC model that mimics the human outer-blood retinal barrier as closely as possible. However, the goal of our current study is to establish well-characterized read-outs that are relevant for clinical translation. Such read-outs will also be essential when studying OOC models of the oBRB that integrate stem cell-derived tissue.

3.3.2 Fluorescein angiography on 3D microvessels in the microfluidic chip

In order to explore the relevant conditions of oxidative stress to cause endothelial dysfunction in our cultured cells, we first treated HUVECs in regular culture systems to different concentrations of H_2O_2 (Suppl.Fig.7.1.1). Here, our logic for using H_2O_2 as a disease stimulus was motivated by the fact that RPE cells generate hydrogen peroxide

during photoreceptor outer segment digestion.⁵⁰ Disrupted metabolic activities by aging may cause accumulation of free radicals in the native microenvironment that eventually damages nearby tissues such as the choroid. It has been reported that hydrogen peroxide is neutralized by HUVECs (1nmol H₂O₂ per 10³ cells per hour)⁵¹, however continuous exposure causes the damage to accumulate. Here, we studied the long-term effects of peroxide exposure on cells.

In ophthalmology, FA is a powerful imaging modality commonly used to assess circulation in the eye (*e.g.* fluid leakage from the choroidal blood vessels) as well as to find vessel defects that are not detectable otherwise.⁵² Therefore, many clinical trials of AMD rely on FA to evaluate the potency of treatments. FA involves intravenous injection of a fluorescent tracer like fluorescein, which then flows through the circulation and is visible by fluorescence imaging within the choroidal microvessels within seconds. After a mid-stage, in which all retinal vessels light up for a few minutes, the dye is gradually eliminated from the system. If a retinal vascular defect or a defect in the RPE is present, the dye fills up the intercellular space from the lesion and is retained even after most of the dye has been cleared from the retinal vasculature and choroid. This remaining hyperfluorescence in the late phase of the angiogram is a clinical indication of barrier tissue damage.⁵³

We combined FA with OOC technology to examine the effect of the previously established concentrations of H₂O₂ (see Supplementary Methods) on intercellular leakage of co-cultures in our model (Fig.3.2). To minimize the possible damaging effect of flow on the cell barrier, we opted for shear rates ($\sim 34\text{s}^{-1}$) much lower than those found in arteries ($\sim 300\text{-}1000\text{s}^{-1}$).⁵⁴ Since the volume of the channels of our chip was much lower than well plate cultures, we made use of a rocking platform in order to maintain cell viability for multiple days in our devices. This platform was able to rotate from side to side allowing cyclic patterns of hydrostatic pressure from the attached reservoirs, and this in turn made it possible for cells to access a larger volume of medium and hydrogen peroxide. In our experiments, we exposed the co-cultures on-chip for 1, 2 and 5 days to concentrations of 800 μM and 10mM H₂O₂, prior to performing the ‘on-chip FA’. The on-chip FA relied on introducing a fluorescently labelled dextran into the patterned microvessel, which could be detected by fluorescence microscopy. After perfusing the system with the dye for a few minutes (Fig.3.2B, ‘Perfusion’), the model was perfused with control medium to clear the dye from the system and subsequently inspected for signs of hyperfluorescence, the late stage (Fig.3.2B, ‘Removal’).

Using fluorescence intensity data from the ‘Perfusion’ phase of the on-chip FA, we calculated the permeability of the endothelial monolayers. For that, first we corrected the images for background intensity, to eliminate the effect of ambient light. Afterwards, fluorescence intensities of the gel were normalized to microvessel intensities. Using these normalized intensities, we calculated the slope of the gradual increase in fluorescence (Fig.3.2C). Normalizing intensities minimized the inconsistencies of the fluorescence intensity of dye perfused through the microvessel, and provided an objective method to compare data from multiple chips and multiple days.

Based on FA analysis of untreated samples, our on-chip cultures maintained their barrier over the course of the treatment (Fig.3.2D). Moreover, 800 μM treatment did not cause significant damage to the barrier in shorter exposures (1 day). However, significant damage occurred upon 2 and 5 days of exposure. As a positive control, we used a very high

concentration of H_2O_2 10mM, which caused significant damage to the cellular barrier after 1, 2 and 5 days of exposure as well. Here, our FA setup was only used for semi-quantitative determination of permeability, as the diffusion time for labeled dextran to travel from the microvessel-gel border to the channel wall ($\sim 8\text{min}$) was longer than the time of the 'Perfusion' phase ($\sim 4\text{min}$). As a result, the gradients within the gel were still significant at the end of this phase. Therefore, any quantitative estimation of the permeability from the rate of change of this average intensity would be significantly skewed. The technical impossibility of full quantification prevents a direct comparison of permeability measurements in our system with other *in vitro* culture systems.

In addition to performing this semi-quantitative analysis of the fluorescence data, our method based on microscopic imaging also allows a qualitative inspection of the microvessels for local defects. Normally, the dye leaks from the microvessel in a uniform pattern, but occasionally, we found patterns of enhanced dye accumulation at specific sites of the microvessel (Suppl.Fig.7.1.3).

In order to reveal the cell morphology upon peroxide exposure, we fluorescently stained HUVECs inside the microvessel for nuclei and actin filaments. In line with our observations in the FA analysis, we observed a dose-dependent effect on the cell morphology and monolayer integrity (Suppl.Fig.7.1.4). At $800\mu\text{M}$ H_2O_2 , cells acquired an elongated morphology with strong F-actin stress fibers and damaged monolayers with scattered intercellular holes. At 10 mM of H_2O_2 , cells displayed a shrunken morphology, and intercellular spaces became so large that the monolayer disappeared almost completely. Interestingly, the observed patterns of monolayer damage (particularly in the condition of $800\mu\text{M}$ H_2O_2) were not as severe as what was observed in our experiments with HUVECs in culture plates, in which we already observed strongly reduced cell numbers upon treatment with $800\mu\text{M}$ H_2O_2 (Suppl.Fig.7.1.1C). This is presumably due to the low internal volume of the chips, which causes reduced cellular exposure to H_2O_2 and its short-lived radical oxygen species compared to culture plates with high absolute amounts of H_2O_2 . Alternatively, endothelial cells in chips could be better protected from damaging stimuli because their culture conditions better recapitulate the native *in vivo* conditions of these cells.

In our experiments with co-cultures, even with high concentrations of H_2O_2 , no considerable damage to the layer of RPE was observed as there was no dye accumulation in the culture chamber. A possible reason for this might be that the effective concentrations of H_2O_2 to which the RPE cells are exposed is strongly reduced because they were shielded from the H_2O_2 in the patterned microvessel by both the cultured endothelium and the collagen hydrogel. Another reason may be the specific RPE cell line we utilized in this study; ARPE-19 has been reported to be highly resistant to oxidative stress.⁵⁵ Even though our current disease stimulus does not have a damaging effect on RPE, we confirmed that our FA analysis is suitable to evaluate this type of damage. For this, we performed experiments in devices with monocultures of endothelial cells only. The absence of the ARPE-19 layer in the culture chamber enabled us to simulate the severe late stages of AMD where both endothelial and epithelial layers are damaged.³ In these experiments, we found leakage of dye towards the culture chamber upon treatment with H_2O_2 (Suppl.Fig.7.1.5)

Our results demonstrate that an FA can be carried out on the OOC co-cultures, and that this method allows both semi-quantitative evaluation of the endothelial barrier by analyzing the

slope of the fluorescence increase in the ‘Perfusion’ phase, as well as qualitative assessment of lesions and defects by analyzing local accumulation of the fluorescent dye in the ‘Removal’ phase.

In the clinic, two dyes are typically used in fluorescence-based angiography: fluorescein and indocyanine. Fluorescein has a low molecular weight (0.3kDa) and therefore readily leaks out of the fenestrated choroidal capillaries, leading to a diffuse ‘choroidal flush’ early in the angiogram that reveals few details about the choroidal vasculature. It is therefore mostly used to find defects in the retinal pigment epithelium and the retinal vasculature. In contrast, indocyanine binds strongly to plasma proteins and is therefore retained in the lumens of the fenestrated choroidal microvessels. Patterns in the indocyanine angiogram like delayed filling of vessels or focal hyperfluorescence can therefore be used to diagnose defects in the choroidal vasculature. For example, hyperpermeability of choroidal vessels is often observed in diseases like central serous retinopathy and AMD.⁵⁶⁻⁶⁰ The dye that we used in our studies was a fluorescein-labeled 40kDa dextran, which diffuses over the endothelial barrier at a rate much lower than fluorescein, but which is not retained fully in the vessels like high molecular weight plasma proteins. These properties allow us to reliably track the diffusion of the dye into the collagen gel over time by time-lapse microscopy, which enables sensitive semi-quantitative analysis of vascular permeability. Fluorescein would diffuse too rapidly to perform a reliable quantification, while a very high-molecular weight dye (*e.g.* fluorescently labeled albumin, 66kDa) would instead only allow qualitative identification of extreme vascular defects. Since our assay gives information on the state of the ‘choroidal’ vessel in the OOC, the observed increase in permeability (Fig.3.2D) upon treatment with hydrogen peroxide can be considered to be analogous to choroidal hyperpermeability observed in clinical indocyanine angiography.

3.3.3 Optical coherence tomography on 3D microvessels in the microfluidic chip

OCT is a method in ophthalmology to assess the structure of the tissue layers of the retina. Because it is a non-invasive readout, and allows for *in vivo* imaging, it is a routine tool for diagnosis and follow-up after AMD treatment. OCT is based on a pattern that is formed by backscattered light from the sample. Through the interference of this pattern with the reference beam (Suppl.Fig.7.1.2), an optical map of the structures of the retina can be drawn.

In this study, we explored whether our OOC model of the outer tissue of the retina is compatible with OCT, and whether structural changes in the on-chip hydrogel structures could be visualized. To characterize the sensitivity of using OCT as a read-out, we first used it to measure patterned microvessels of defined, but different sizes: 500, 300 and 120 μ m. Horizontal and vertical axes lengths showed a low variance and average lengths were approximately the same size as the intended diameters (Fig.3.3B). In addition, we evaluated the quality of these microvessels, Q_{vessel} (see Materials and Methods), using OCT data. Consistent with our optical observations, analysis of vessel quality revealed a higher number of microvessels in good quality ($Q_{\text{vessel}} > 0.5$) for larger sized (500 μ m) patterned microvessels (high quality: 15/32), as compared to smaller ones: 5/48 and 1/32 for 300, 120 μ m microvessels, respectively (Fig.3.3C-i). Any microvessel below Q_{vessel} of 0.5 was deemed improper as these were partially destroyed and non-perfusable (Fig.3.3C-ii,iii).

We then set out to study whether OCT can not only be used for analysis of pre-patterned microvessels, but for self-developed microvessels of physiological sizes as well. For this, we compared our regular OOC with those in which the collagen I hydrogel was replaced with a fibroblast-containing fibrin hydrogel. It is known from literature that fibrin hydrogels can strongly induce cultured endothelial cells to form network branches *in vitro*.⁶¹⁻⁶⁴ After 7 days of culturing, the matrix (Fig.3.3D) in our OOCs was imaged by OCT. Typical cross-sectional scans of the patterned microvessel in a collagen I matrix revealed a lumen with the same cross-section as the needle used for patterning, with clearly delineated borders (Fig.3.3D- i). In contrast, scans of the fibrin gel (Fig.3.3D- ii) revealed an enlarged lumen and a damaged matrix structure with cavities (Fig.3.3D- ii, white arrows), which coincided with endothelial growth into the fibrin hydrogel (Suppl.Fig.7.1.2C). Here it is worth noting that the resolution was considerably lower in the upper part of the damaged matrix due to the thick PDMS layer below the microchannel. By analyzing the OCT scans, we determined the size distribution of the observed cavities. The majority of cavities consisted of smaller vessels (~20µm, Fig.3.3D-iii), sizes which are consistent with choroidal capillaries *in vivo*⁶⁵. Note that because of the 3D acquisition scheme described in Materials and Methods, artefacts were prone to appear in OCT images (Fig.3.3D), due to specular reflections and incomplete removal of DC component in the signal which were consistent with recent relevant studies^{66, 67}.

Our data demonstrate that OCT can be used to visualize the physical structure of the matrix, as well as formation of new microvessels in our OOC device. The resolution of the imaging technique is high enough to not only detect large structural defects (Fig.3.3C-iii), but also microvessels and cavities of physiological sizes (Fig.3.3D-iii). Given the importance of OCT as a clinical tool, this read-out should in the future enable the comparison and correlation of data from our *in vitro* assay with clinical observations. In the clinic, OCT provides information about structural defects in the RPE in the form of drusen formation and fluid accumulation, as well as neovascularization from the choroid.⁶⁸ Currently, the lack of resolution in the area of RPE culture in our device prevents us from screening for sub-retinal fluid accumulation or drusen formation. However, the technique does allow us to observe changes in capillary density and neovascularization, which are key steps in the pathophysiology of AMD preceding RPE dysfunction and photoreceptor degradation.⁶⁹ Future studies will focus on improving the imaging depth of the set-up.

3.4 Conclusion

Microfluidic OOC systems have great potential to investigate basic mechanisms of disease pathology and organ-level physiology due to their flexibility of incorporating various cell types and physiologically relevant biochemical readouts.²² In addition, they can be used to test the effect of disease triggers and drugs. Here, we report an OOC model of the oBRB, and we use it to track pathophysiological processes relevant for AMD.

Our model consists of a microvessel that is defined by patterning a collagen I hydrogel to recapitulate the *in vivo* choroidal microenvironment. Using H₂O₂ to mimic oxidative stress, one of the well-known disease factors in AMD pathophysiology, we explored the effects on co-cultures in our OOC devices. Using a readout that is analogous to the clinically used FA, we showed that on-chip co-cultures were affected by the H₂O₂ stimulus. In addition, we used OCT to image the hydrogel matrix of our OOC model, as well as 3D microvascular

structures formed by cells within the hydrogel. Together, our results demonstrate the added value of integrating relevant read-outs in complex *in vitro* models like OOCs when studying the oBRB.

There are various challenges in investigating AMD pathophysiology *in vivo* due to AMD being a multifactorial disease.⁵ The model, including read-outs, presented in this study demonstrates the potential added value of using OOCs for future studies of disease mechanisms and treatment development for AMD.

As a next step, co-cultures of patient stem cell-derived endothelial cells and RPE cells can be integrated into the chip to generate a more representative model of AMD. Moreover, the integration of clinically relevant read-outs in our model will strongly facilitate future side-by-side comparison of *in vitro* findings with patient data. Together, this would make our OOC model of the outer tissues of the retina a powerful functional test for evaluating potential treatments for specific patients or patient sub-groups in the context of precision medicine.⁷⁰

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Collagen I Based Enzymatically Degradable Membranes for Organ-on-a-chip Barrier Models

Organs-on-chips (OOC) are microphysiological *in vitro* models of human organs and tissues that rely on culturing cells in a well-controlled microenvironment that has been engineered to include key physical and biochemical parameters. Some systems contain a single perfused microfluidic channel or a patterned hydrogel, whereas more complex devices typically employ two or more microchannels that are separated by a porous membrane, simulating the tissue interface found in many organ subunits. The membranes are typically made of synthetic and biologically inert materials that are then coated with extracellular matrix (ECM) molecules to enhance cell attachment. However, the majority of the material remains foreign and fails to recapitulate the native microenvironment of the barrier tissue. Here we study microfluidic devices that integrate a vitrified membrane made of collagen I hydrogel (VC). Biocompatibility of this membrane was confirmed by growing a healthy population of stem cell derived endothelial cells (iPSC-EC) and immortalized retinal pigment epithelium (ARPE-19) on it, and assessing morphology by fluorescence microscopy. Moreover, VC membranes were subjected to biochemical degradation using collagenase II. The effects of this biochemical degradation were characterized by the permeability changes to fluorescein. Topographical changes on the VC membrane after enzymatic degradation were also analyzed using scanning electron microscopy. Altogether, we present a dynamically bio-responsive membrane integrated in an OOC device, with which disease-related ECM remodeling can be studied.

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4.1 Introduction

The extracellular matrix (ECM) is the non-cellular component of tissues and organs. Not only does it provide physical support to cells, but it initiates biochemical and biomechanical cues.¹ ECM is composed of a great variety of molecules such as collagen-family proteins, glycosaminoglycans, proteoglycans and adhesive glycoproteins. Different organization of these components gives rise to different tissue characteristics. Collagens, in particular type I, II and III, are the most abundant proteins in the human body.² Collagens are responsible for key tissue-level functions such as cell attachment and spreading, in addition to mechanical and structural functions. Furthermore, these properties have an influence on cellular differentiation and movement.³ The importance of ECM is also illustrated by the wide variety of diseases that arise from genetic abnormalities in ECM proteins.⁴

Considering the significance of the ECM in fundamental cellular processes and disease pathologies⁴, experimental models where changes to tissues can easily be observed and experimental conditions can be manipulated are needed. In recent years, microfluidic OOC devices have been proven to be promising in *in vitro* disease modelling platforms that recapitulate human specific physiology.⁵⁻¹² These devices are miniaturized cell culture platforms comprised of defined microchannels which are inhabited by living cells to mimic tissue-organ level physiology. Depending on the research question, physico-chemical parameters of the native tissue environment can be incorporated. While simple devices contain only one type of cell cultured in a perfusable chamber, more complex devices have multiple channels separated by semi-permeable porous membranes lined by two or more type of cells.

These semi-permeable membranes located between adjacent culturing chambers in OOCs aim to mimic the basement membrane, a type of ECM creating boundaries between tissues. Moreover, these membranes provide physical anchoring points for cells while enabling compartmentalization. This is due to its porous structure which prevents cell migration and allows exchange of soluble signaling cues through the pores. Despite being widely used in OOCs, porous membranes are usually made of synthetic polymers (*e.g.* poly-dimethyl siloxane (PDMS), polycarbonate, polyester) which differ from ECM found *in vivo*. To render the membranes more bioactive, they are often coated with ECM-proteins (*e.g.* fibronectin, collagen, laminin or Matrigel®) to enhance attachment of anchorage-dependent cells to the membrane surface^{13, 14}. Despite these efforts, the majority of the material remains synthetic and fails at mimicking biochemical cues that affect structure and function of cells as well as the fibrillar ultrastructure of basement membranes.¹⁵ Furthermore, custom fabrication of synthetic membranes from alternative materials or with engineered properties requires dedicated systems such as track etching,¹⁶ chemical etching,¹⁷ phase inversion¹⁸ and electrospinning.¹⁹

To address these challenges, previous studies used either *ex vivo* basement membranes or vitrified membranes fabricated using different configurations of natural hydrogels.²⁰⁻²⁹ For example, Mondrinos *et al.*²¹ reported membranes that uses three-step fabrication (gelation, dehydration, and vitrification). Resulting membranes are thin, fibrillar and stable enough to be incorporated into a microfluidic device. Although these studies demonstrate the feasibility of integrating these membranes in microfluidic chips, none of them provide full characterization of the incorporated membranes in terms of ultrastructure, enzymatic

Figure 4.1 Fabrication of vitrified collagen membrane and OOC device in which the membranes were integrated. (A) Vitrified collagen membranes were fabricated by depositing a neutralized collagen solution on a PDMS slab with defined rectangular shapes, which was subsequently dried in aseptic conditions. This resulted in a thin film of collagen along with salts and other phenol red. Following drying, the collagen film was washed with deionized water to remove salts and phenol red. After a second drying process, a thin film of collagen was obtained, which was easily handled and could be incorporated into the OOC device. (B) PDMS based OOC device with exploded view (B-left) and assembled final device (B-right). The device contains a 1mm² square microchannel (B-i), at the center of which the membrane was located. There is an open-top culture chamber (3mm Ø) situated above the membrane (B-iii). PDMS layers were assembled by applying mortar to surfaces (blue, I and III) to sandwich the membrane in between. (C) Injection molding was used in order to eliminate the labor intensive fabrication procedure. (C- left) Different layers of the device were assembled by incorporating the collagen I based membranes in between the channels held by magnets on each end. (C-middle) Final assembled device consists of a PDMS-coated glass cover slip, a square microchannel, collagen based membrane stretched in the center, another square microchannel on top. Final assembled layer only requires plasma activation of surfaces to be attached to glass coverslip (C- right).

degradation and dynamic permeability or simplification of the integration process in OOCs.

Here, we report a collagen I based membrane incorporated in an OOC device. In our study, we study membrane ultrastructure and permeability, as well as adhesion of both endothelial and epithelial cells. Moreover, we characterize the degradation and remodeling of the basement membrane by a protease. In addition, we provide an injection molding design to fabricate our devices, which eliminates the labor intensive utilization of the toxic PDMS mortar to incorporate the membranes between channels. Our study reinforces the notion that vitrified collagen membranes have strong added value in OOC engineering.

4.2 Experimental Section

4.2.1 Membrane Fabrication

Collagen I based membranes were prepared via multistep procedure depicted in Fig.4.1A. First, rat tail collagen type I (VWR) was prepared according to the manufacturer's

Figure 4.2 Collagen based membranes are biocompatible and allow for adhesion and formation of monolayers of cells. Immunolabeling of cells on collagen-based membranes revealed a continuous distribution of each cell type indicated by DAPI (nuclei) and actin filament staining. These cells were positive for their respective cell-cell adhesion markers: VE-cadherin expression for hiPSC-EC and ZO-1 expression for ARPE-19, inset showing the highlighted area. Scale bars: 50 μ m. Following incorporation to devices, membranes were enzymatically treated. First, collagenase II was diluted in PBS to a desired concentration (24, 48, 120U/ml) and pipetted onto membranes. Afterwards, devices were incubated for 5min at 37°C with 5% CO₂. Following treatment, collagenase II was removed from culture chamber, and membranes were washed with PBS to remove any remaining enzyme. All experiments were performed at least twice.

instructions at a concentration of 3mg/ml and a pH between 7.5 and 8 by mixing with dH₂O, phosphate buffered saline (PBS, ThermoFisher) and 1M Sodium hydroxide solution. Afterwards, the collagen solution was pipetted evenly onto a PDMS slab (0.25ml/cm²). This was followed by overnight dehydration in aseptic conditions at room temperature (RT). Evaporation of water resulted in a thin film of collagen on the surface of PDMS. Subsequently, this collagen film was rehydrated with dH₂O for 4h at RT in order to remove salts and other impurities.

Membranes underwent another drying cycle after gentle aspiration of water. Following this second dehydration step, membranes were cut into chip sized pieces (~6mm²). To generate multi-layered membranes, collagen membranes with 3 and 4mg/ml collagen-I were prepared as mentioned. Second layer of membrane was placed onto the first layer following the addition of 10mU/ml transglutaminase (Ajinomoto) in PBS onto the first dehydrated membrane for 2h at 37°C. Following incubation, membranes were washed 3 times with PBS. Cross-sections of membranes were done by first immersing the double layered membranes in liquid nitrogen until they are completely frozen and manual breaking the layers afterwards.

4.2.2 Chip Fabrication

Casted poly (methyl methacrylate) (PMMA, Altuglass) master molds containing channel imprints were first designed using Solidworks and fabricated with a computer numerical control (CNC) milling machine (Datron Neo, Datron AG). Afterwards, PDMS base and curing agent were mixed at a ratio of 10:1 (wt: wt) (Sylgard 184 Silicone elastomer kit, Dow Corning). It was then degassed and poured onto the positive PMMA molds and subsequently cured for at least 3h at 65°C. After that, cured PDMS was separated from the molds as slabs. PDMS slabs with square microchannels (1mm²) were cut from each side to generate side inlets (Fig.4.1B-i).

Figure 4.3 Characterization of collagen membranes following enzymatic treatment. (A) SEM images of membrane structure that are untreated or treated with various concentrations of collagenase II (24, 48 and 120U/ml). Scale bars: 2μm. (B) Distribution of fiber diameters of enzymatically treated membranes (n=400 for each condition).

After that, 2 inlets and 1 culture chamber (1.2mm and 3mm in diameter respectively) were punched into the PDMS slab corresponding to the middle part of the final assembled device (Fig.4.1B-iii). Furthermore, 3 reservoirs were punched (5mm in diameter) into another PDMS slab for the top compartment of the assembled device (Fig.4.1B-iv). Subsequently, all three slabs were aligned and cut into device-sized pieces. Prior to assembly of the device parts, dust was removed by Scotch tape (3M). Leak-free assembly of the parts was achieved by using uncured PDMS/toluene mortar (5:3 wt ratio) (toluene from Merck) as previously reported.^{30, 31} First, this mixture was spin-coated onto a glass coverslip (1500rpm, 60s, 1000rpm/s, Spin150, Polos) and transferred to the device parts with an ink roller. Second, membranes were cut into small squares (~36mm²) (Fig.4.1B-Membrane), aligned and sandwiched between the center of the bottom (Fig.4.1B-i) and middle (Fig.4.1B-iii) compartments. Afterwards, assembled parts were baked overnight at 65°C. Finally, the surfaces of the top compartment and the pre-assembled device were exposed to air plasma (50W) for 40s (Cute, Femto Science). After plasma treatment, activated surfaces were pressed together to complete the assembly of the device.

In addition to mortar-assembled devices, injection molding was used to incorporate membranes. First, collagen membranes were wetted to attach to top channel imprints in PMMA molds. This was followed by closing the bottom mold and applying four magnets

per side from each end to hold the membrane in between during PDMS injection. Afterwards, PDMS was freshly prepared and mixed as aforementioned with additional carbon powder (1% wt:vol ratio, Vulcan XC-72R, Fuel Cell Store). The mixture was then injected through the inlet of the mold using a syringe (Norm-Ject, Henke Sass Wolf). As soon as the mold was filled with PDMS, syringe was removed and mold inlet was sealed with cured PDMS. Final mold was first incubated for 30min at RT to eliminate any air bubbles, then was baked for at least 3h at 65°C.

4.2.3 Permeability Assay

On-chip: The permeability of vitrified collagen membranes was measured by means of fluorescein diffusion. First, the culture chamber (Fig.4.1B-iii) was filled with phosphate buffered saline (PBS, ThermoFisher). Second, 200µg/ml fluorescein sodium salt (0.3kDa, SigmaAldrich) diluted in PBS was pipetted into the microchannel (Fig.4.1B-i). After that, from the beginning of the experiment, a sample of 5µl was collected from the culture chamber every 15min and the levels were normalized by adding PBS to the culture chamber. To ensure homogeneity of the dye concentration along the microchannel, dye transferred from one inlet to the other after every sampling. To avoid flow from the microchannel to the culture chamber, fluid levels in the culture chamber were maintained at the same level as the highest inlet (Suppl.Fig.7.2.1A-C).

These samples were read by a plate reader (Victor3, Perkin Elmer). Using a standard curve, fluorescence values were matched with concentrations. The permeability (P_{membrane}) of membranes was calculated by:

$$P_{\text{membrane}} = \frac{dc}{dt} \times \frac{\text{Volume}_{\text{chamber}}}{C_i \times \text{Area}_{\text{chamber}}} \quad (4.1)$$

With P_{membrane} the permeability in cm/s, C_i the initial fluorescein concentration in µg/ml. $\text{Area}_{\text{chamber}}$ and $\text{Volume}_{\text{chamber}}$ are the dimensions of the culture chamber (in cm² and cm³ respectively) and dC/dt was the change in the concentration (µg/ml·s). Permeability measurements were repeated following enzymatic treatment of membranes.

Transwell: On-chip permeability assay with synthetic membranes were compared with conventional Transwell systems. The levels of solutions in both Transwell compartments were equalized to avoid pressure-driven fluid flow. While the bottom well was filled with PBS, the insert was filled with 200µg/ml fluorescein. Afterwards, the permeability assay was carried out as described in the section above (Suppl.Fig.7.2.1B-C).

4.2.4 Imaging of Membranes

Prior to imaging, membranes were fixed with fixation buffer which contains 2% paraformaldehyde (Sigma Aldrich), 2.5% glutaraldehyde (Sigma Aldrich) in 0.1M sodium cacodylate buffer (Sodium cacodylate trihydrate in ultrapure H₂O at pH 7.4) overnight at 4°C. Fixation buffer was gently aspirated and membranes were washed 3 times with cacodylate buffer. After that, membranes were treated with 2% osmium tetroxide solution in sodium cacodylate buffer for 1h at RT. After osmium tetroxide treatment, membranes were washed again with sodium cacodylate buffer 3 times. Afterwards, membranes were dehydrated progressively by submerging membranes in ethanol with increasing concentrations (70, 80, 90 and 100%) for 5min at RT. This was followed by drying membranes with a critical drying point apparatus (Leica EM CPD030) according to

manufacturer's instructions. Following fixation, membranes were sputtered with gold (Sputter Coater 108auto, Cressington Scientific Instruments) for imaging with scanning electron microscope (SEM, JSM-IT100, JEOL).

4.2.5 Cell Culture

Human immortalized retinal pigment epithelial cells (ARPE-19, ATCC) were cultured with DMEM/F12 (with GlutaMAX, ThermoFisher) supplemented with 10% fetal bovine serum (FBS) and 50U/ml penicillin/streptomycin (P/S). ARPE-19 cells were cultured in non-coated T75 flasks. Human iPSC derived endothelial cells (hiPSC-EC) were derived from a healthy control hiPSC line as described previously³². hiPSC-EC were cultured in human endothelial serum free medium (ThermoFisher) supplemented with 1% platelet poor plasma derived serum (BioQuote), 0.6 μ g/ml VEGF (R&D Systems) and 0.2 μ g/ml FGF (Miltenyi) in collagen coated flasks (0.1mg/ml). The cells were incubated at 37°C in humidified air with 5% CO₂. Flasks with confluent monolayers were either used for experiments or subcultured. ARPE-19 and hiPSC-EC were kept in culture up to passage number 30 and 4 respectively.

Prior to staining, ARPE-19 and hiPSC-EC cells were seeded on membranes. hiPSC-EC and ARPE-19 cells were obtained from a confluent flask using 1x Tryple (ThermoFisher) and 0.05% Trypsin-EDTA (ThermoFisher) respectively.

Figure 4.4 Collagen membranes were characterized in terms of permeability. (A) Permeability of various membranes were measured: Polycarbonate transwell membrane (PC Transwell, $n=4$), polyester (PE, $n=16$), collagen (UT, $n=100$), enzymatically treated (Col-2+, $n=9$) membranes. Significant differences ($p<0.05$, Student's t -test) are denoted by an asterisk. (B) Effect of cell seeding on collagen membranes by means of permeability. Non-treated collagen membranes (UT, $n=100$), non-treated membranes with ARPE-19 cells seeded (UT+ ARPE-19, $n=5$), and enzyme treated membranes (Col-2+ ARPE-19, $n=5$) were measured. Significant differences according to one-way ANOVA and Post-hoc Tukey's tests are indicated by asterisks.

4.2.6 Cell Staining

Cells cultured on membranes were stained for cell specific adhesion markers, actin filaments and nuclei for confirmation of cell monolayers and their health. First, cells were washed with PBS and fixed with 4% formaldehyde (in PBS, ThermoFisher) for 15min at RT. Following fixation, cells were washed 3 times with PBS. After that, cells were permeabilized for 60min at RT with permeabilization buffer (PB), which contains 0.1% Triton X-100 (Sigma Aldrich) and 10mg/ml bovine serum albumin (BSA) in PBS.

Afterwards, ARPE-19 were incubated with mouse anti-human ZO-1 IgG (5µg/ml in PB, BD Transduction Laboratories) for 2h at RT. Following incubation, the cells were rinsed three times with PBS and washed three times with PBS for 10min at RT. After that, the cells were incubated with 1.25µg/ml 4',6-Diamino-2-Phenylindole (DAPI, ThermoFisher), 2drops/ml ActinGreen (binds to actin filaments, ThermoFisher), donkey anti-mouse IgG Alexa Fluor 647 (5µg/ml, ThermoFisher) in PB for 1h at RT.

The cells were imaged with phase contrast, fluorescence microscopy using the EVOS FL Cell Imaging System (Life Technologies; GFP filter (ex 470/22 em 510/42) for ActinGreen, Cy5 filter (ex 628/40 em 692/40) for ZO-1 and DAPI filter (ex 357/44 em 447/60) for DAPI).

4.3 Results And Discussion

4.3.1 Integration of membranes to devices

Conventionally, integration of membranes to OOC devices, depending on the type of membranes require plasma activation of surfaces or application of PDMS mortar to adjacent surfaces between of which membranes are incorporated³³. Using a PDMS mortar in these devices requires the assembly of each device separately which in turn increases the fabrication time. Here, in addition to mortar-based assembly of devices, we utilize injection molding as an alternative to conventional fabrication method. This eliminates the separate assembly of devices and significantly reduces the fabrication time (Fig.4.1C, Suppl.Fig.7.2.5A). Integrated membranes are held between the channels by magnets from both ends. Resulting membranes do not contain any PDMS residues that is indicated by the absence of black PDMS on the membrane (Suppl.Fig.7.2.5B).

4.3.2 Production of Collagen I Based Bio-responsive Membranes

In this study we aim to mimic the native *in vivo* tissue interface provided by basal membranes. As the material of our membranes we chose collagen type I, since it is the most abundant protein in the human body. Moreover, one of the components of the basement membrane, basal lamina anchors to the adjacent connective tissue using networks of type I collagen fibers in the reticular lamina.^{21, 34} In addition, type I collagen is also found in specialized membranes such as Bruch's membrane which facilitates nutrient/waste exchange between retinal pigment epithelium and choroidal capillaries in the retina and provide structural support for adjacent tissues.³⁵

The fabrication process of the membrane relied on drying collagen I solutions on a non-adhesive, PDMS surface. Fabrication did not require dedicated equipment and resulted in membranes with a thickness of ~2µm and a fibrillar structure (Fig.4.3A-I, Suppl.Fig.7.2.4A). Membranes were prepared using a concentration of 3mg/ml collagen I in the original solution. Lower concentrations resulted in membranes that were not sturdy enough to be handled.

In addition to single layered membranes, we also fabricated double-layered membranes using membranes with collagen concentrations of 4 and 3mg/ml. Cross-sectioning of these membranes were proven to be challenging as the fibrous structure does not separate well enough using the freeze fracture method, however multi-layered structure can be deduced (Suppl.Fig.7.2.4B).

These membranes also allow for cell culturing as evident in Fig.4.2. We obtained monolayers of cells lining the membrane surface indicated by the homogeneous distribution of nuclei and actin cytoskeleton stainings (Fig.4.2). Moreover, these cells grow to a healthy population as evident by their respective cell-cell adhesion markers (VE-cadherin for hiPSC-ECs, ZO-1 for ARPE-19). A healthy morphology can also be seen when these cells were seeded to glass coverslips, and express their respective markers (Suppl.Fig.7.2.6). According to the manufacturer's information, collagen I from rat tail was isolated using acetic acid extraction without any further enzymatic treatment. As a result, in the final isolated proteins, telopeptide domains are intact. It has been reported by studies using porcine or bovine collagen that may illicit an immune response in planted microdevices or scaffolds in humans even though this is rare.³⁶ Moreover, to our information a potential immune response to rat tail collagen was not reported by the manufacturer, and this do not cause any issues regarding a decrease in viability and attachment of cells.

4.3.3 Enzymatic Degradation of Membranes

During embryonic development, as well as in disease processes, multiple cell types traverse the barriers of the basal lamina.³⁷ These transmutations are often associated with proteases, a class of enzymes responsible for remodeling the basement membranes. This has been supported by the observation of irreversible changes to the ECM during tissue-invasive events that are associated with development and disease states.³⁷ For example, gaps in the basement membrane have been identified at sites of cancer invasion *in vivo*.³⁷⁻⁴⁰ In addition, an increased expression of ECM degrading proteases has been observed in neoplastic epithelial cells.⁴¹⁻⁴³ Moreover, in retinal diseases such as the wet form of age-related macular degeneration, choroidal capillaries penetrate the Bruch's membrane and grow into the retina, leading to leakage of the contents which leads to blindness. In patients, the expression of matrix remodeling enzymes are elevated.⁴⁴ These enzymes secreted by surrounding vascular endothelium and macrophages degrade extracellular matrix which allows infiltration of Bruch's membrane by the adjacent capillaries.^{45, 46} Given the importance of basement membrane remodeling in development and disease, we set out to study the enzymatic remodeling of our vitrified collagen membranes.

We treated the membranes with collagenase II to affect their structure and properties. We selected this enzyme because it has been used in tissue dissociation and is extensively characterized.⁴⁷ Due to its potency in tissue dissociation, we exposed the membranes to relatively low concentrations (24, 48, or 120U/ml) for a short duration of 5min. Membranes that were treated longer or with higher enzyme concentrations became unstable and easily fractured during permeability measurements (Suppl.Fig.7.2.2).

Fiber Diameter: SEM imaging of membranes treated with collagenase showed striking differences in morphology between treatment conditions. Single collagen I fibers can easily be distinguished from one another in the untreated membranes (Fig.4.3A-i). However, upon treatment with increasing concentrations of collagenase this clarity is lost (Fig.4.3A-ii-iv). Moreover, there is a dose-dependent trend in terms of fiber diameters when compared to untreated membranes. In treated membranes, the majority of fibers have a diameter of 50nm, in contrast to untreated membranes in which diameters exhibited a broad distribution from 50 to 400nm (Fig.4.3B). This observation is in line with existing information about the fiber size as it ranges between 50 and 200nm in diameter.⁴⁸ The morphological changes upon treatment with collagenase are in line with its mechanism of action, which depends

on cleavage of the triple helix of collagen at multiple sites. As a result, fiber thinning occurs.
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Membrane Permeability: Considering the importance of diffusion of solutes across a barrier in modelling barrier tissues that many OOC platforms tackle, we characterized the membranes in terms of permeability by measuring the diffusion of fluorescein (Fig.4.4). First, we measured the permeability on membranes in which cells were not cultured. Based on these measurements, collagen membranes showed a comparable permeability to polyester membranes which we incorporated in our devices (Fig.4.4A). Apparent permeability values of membranes in our devices were significantly lower overall than the polycarbonate membranes in Transwell inserts (Fig.4.4A). This difference might be due to minor advective transport due to a pressure gradient over the membranes in the devices. Here it is worth noting that the polycarbonate and polyester membranes have the same pore density and pore size. In addition, upon treatment with collagenase, membrane permeability was significantly lowered (Fig.4.4A). This might be due to the partial degradation of collagen fibers, with the degraded material forming a gelatin hydrogel. This would decrease the open porous structure and thereby lower the amount of fully open paths between fibers through which fluorescein can diffuse. In addition to this decreased porosity, one of the collagenase isoforms present in our enzyme, clostripain, can act as a transpeptidase, which may crosslink the fiber fragments resulting in the formation of a gelatin film.⁴⁹ Higher collagenase concentrations or treatment times caused membranes to burst or puncture, thus they were not taken into consideration for permeability measurements (Suppl.Fig.7.2.2).

As a next step, we measured the permeability of membranes that were incorporated in the devices and on which ARPE-19 cells were cultured. We performed the permeability measurements after 3 days of culturing to ensure a healthy monolayer on the membranes (Suppl.Fig.7.2.3). Here, according to our measurements, ARPE-19 growing on membranes significantly lowered the permeability (Fig.4.4B), indicating the formation of a tight monolayer of these epithelial cells. Upon treatment of these cultures with collagenase, no significant effect on the permeability was observed, presumably because the ARPE-19 monolayer shields the membrane from the soluble enzyme (Fig.4.4B).

This result also highlights the fact that cells are the main diffusion barrier to small molecules just as is the case in healthy *in vivo* situation, and that the VC membrane does not significantly interfere in the diffusion process.

4.4 Conclusion

As current cellular and animal models fail at fully recapitulating the *in vivo* microenvironment of human organs and tissues, OOC systems have great potential in investigating disease pathology and organ-level physiology. These systems incorporate various cell types from the human body as well as clinically relevant readouts. To that end, we aimed to eliminate the usage of synthetic membranes as they are not an actual part of the *in vivo* ECM. Here we reported a collagen I based membrane that can be incorporated in OOCs and which we characterized in terms of cell adhesion, ultrastructure and permeability. We demonstrated that these membranes can be treated with proteases, and changes in fiber thickness and permeability can be evaluated. Our results provide actual quantitative permeability values which can be compared with future studies.

As a next step, different ECM proteins like collagen IV and laminin can be integrated into our membrane fabrication to generate an even more representative model of the basement membrane.

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5

Understanding Age-Related Macular Degeneration: An Attempt to Mimic the Inflammation and Anti- Inflammation Mechanism of Endothelial Cells

Age-related macular degeneration (AMD) is a progressive eye disorder that prevents people from performing simple everyday tasks. It affects the macula, a retinal region which captures the greatest focus of the external light. AMD is considered multifactorial with main risk factors being increased age, and genetic predispositions. Risk of AMD increases with several environmental or epigenetic factors such as smoking, diet, and light exposure. These risk factors put the aging retina under chronic stress by the presence of free radicals and oxidized lipoproteins which result in local triggers for para-inflammation. A growing body of experimental and clinical evidence strongly suggests that there is a role of immunological processes in AMD occurrence, consisting of proinflammatory molecule secretion, recruitment of macrophages, complement activation, microglial activation and accumulation in the macular region of the retina where AMD originates. Considering the involvement of the immune system in AMD, new treatment regimens could potentially target inflammatory factors. According to anecdotal evidence, symptoms related to wet-AMD of about 10 patients have been alleviated using two over-the-counter drugs, aescin (anti-inflammatory) and cetirizine (anti-histaminic). This new type of treatment is affordable, stress-free and easy to administer which could be revolutionary for the treatment of AMD. Here in this chapter, we discuss the presumed mechanism of these drugs against the detrimental effects of $\text{TNF-}\alpha$ and histamine using the changed morphology of endothelial cells as a readout. We believe that the arguments raised in this chapter will shed light into further studies into developing treatments for wet-AMD.

5.1 Introduction

Age-related macular degeneration (AMD) originates in the *macula lutea* of the retina, which is responsible for central vision with high visual acuity. There are several recognizable features of the human macula: a neural retina which is composed by the inner neurosensory layer and outer photoreceptor cell layer, underlined with retinal pigment epithelium (RPE). RPE is separated from the choroidal capillaries by Bruch's membrane, a modified basement membrane. The center of the macula called *fovea centralis* is an avascular region and completely inhabited by cone photoreceptors.¹ Due to high metabolic demands, the macula is prone to dysfunction when exposed to stress or over the course of aging.

An individual is at risk of developing AMD due to a wide variety of processes with aging being the primary determinant. Other environmental factors such as history of smoking, diet and phototoxic light exposure significantly increase the risk of AMD occurrence.²⁻⁴ Furthermore, polymorphisms in immunologic/inflammatory genes were shown to correlate with AMD occurrence.^{5, 6} This clearly points to an important role of inflammatory and immune-mediated processes (e.g. complement activation) in AMD pathology. Although AMD is not considered a classic inflammatory or immune disease, immunocompetent cells (*i.e.* macrophages and lymphocytes) are found in affected tissues in AMD.⁷⁻¹⁰

In AMD progression, a process called para-inflammation is in effect. This process is defined as the tissue adaptive response to detrimental stress or malfunction and it is an intermediate state between normal/basal and inflammatory/acute states.^{1, 11} Although, para-inflammation is normally aimed towards preserving homeostasis and restoring tissue function, when tissue is exposed to prolonged stress or malfunction, it is linked in both initiation and progression of many human age-related disorders such as AMD.¹² Even though the clinical pattern of AMD is heterogeneous, para-inflammation is implicated in every form of AMD, including the advanced stage of choroidal neovascularization (CNV) in neovascular AMD.¹³ Para-inflammatory modifications in the retinal tissues related to aging are the breakdown of blood-retinal barrier, activation and subretinal migration of microglial cells in the neuro-retinal structure, an increased number of activated macrophages, morphological abnormalities of melanocytes, choroidal thickening and fibrosis in the choroid.¹² At the interface between RPE and choroid, these changes are particularly demonstrated by complement activation and sub-retinal accumulation of microglia.^{12, 13} Presumably, these events take place because of the aging, combined with accumulating disease stimuli over the decades in the microenvironment. As a result, AMD and CNV progression initially starts as a wound healing attempt in macula.¹⁴

The relation of CNV to wound healing and inflammation is demonstrated by the involvement of several molecules and cells. First of all, during CNV, RPE expresses mediators (e.g. transforming growth factor- β (TGF- β), fibroblast growth factor-1,2 (FGF-1, 2) and tumor necrosis factor- α (TNF- α)) that are also abundant in both inflammatory and wound healing processes. In addition, vascular endothelial growth factor (VEGF) is released by RPE and macrophages which facilitates the progression of CNV.^{13, 15} Secondly, as the insult initiated by the metabolic imbalance, hypoxia and/or accumulation of reactive oxygen species, induces further cellular damage within the outer retinal microenvironment and RPE – Bruch's membrane interface. Due to the damage, release of growth factors (*i.e.* platelet derived growth factor (PDGF) and transforming growth factor) recruits macrophages to the microenvironment, which then release other growth factors (*i.e.* FGF-

2, TGF, PDGF) and additional amount of proinflammatory cytokines (*i.e.* Interleukin-1, TNF- α) which lead to a further VEGF secretion.¹⁴ This causes choroidal endothelial cells to form shoots of capillaries which then can cross Bruch's membrane locally through "damaged" regions by the secreted matrix metalloproteinases (MMP) from the recruited macrophages.¹⁶

In addition to inflammation further stimulated by RPE and macrophages, there are resident inflammatory cells in the choroid called mast cells.^{17, 18} During AMD progression, the number and activation of mast cells increase in the choroid.^{10, 18} As this is observed in all forms of AMD, including early stages, suggests a direct involvement of mast cell activation in the disease progression as well. Mast cells that are activated due to the aforementioned stimuli release cytokines, chemokines as well as histamine into the microenvironment. Together with the release of proinflammatory cytokines from macrophages, activated mast cells intensify and prolong the inflammatory response.¹⁸ Release of histamine induces more mast cells to the environment which might favor the establishment of a chronic inflammatory response, exacerbating the para-inflammation to a severe stage. Histamine also binds to endothelial cells via histamine receptor 1, which increases their permeability causing other residing immune cells to pass through and contribute to inflammation or vessels to leak their contents which in turn leads to the degeneration of photoreceptors.¹⁸⁻²⁰

Considering the involvement of inflammatory processes in the pathology of AMD, it may be advisable to include agents targeted towards complement system or inflammation in the treatment regimen of patients. Furthermore, the presence of variable mid-term responsiveness to the conventional neovascular AMD treatments (anti-VEGF or photodynamic therapy with verteporfin) indicates the need for a combination therapy with drugs directed against the inflammatory response.¹ This notion has been supported by the anecdotal evidence of Prof. Dr. ir. Piet Bergveld and about 10 other AMD patients, that two over the counter drugs, aescin (anti-inflammatory) and cetirizine (anti-histaminic) do have an effect in slowing and eventually stopping the progression of the disease. The effect presumably normalizes the leaky state of the blood vessels, decreasing the severity of the inflammatory state, so that the host immune system can work in maintaining the homeostasis.

Here, in this part of the study, we aimed to perform experiments in model systems to further explore the hypothesis whether these drugs actually work in normalizing the para-inflammatory state of endothelial cells. Cetirizine is a rapidly working, histamine H1 receptor antagonist, which acts primarily on smooth muscle cells, vascular endothelial cells and immune cells.²¹ Given its antagonism, it reverses many of the effects of histamine such as exerting significant anti-inflammatory activity and reducing the infiltration of immune cells.²² On the other hand, aescin is extracted from horse chestnut and possesses anti-inflammatory properties. It has been previously reported that aescin conserves ATP during hypoxia, decreases the histamine response and cytokine release, reduces serotonin induced capillary hyperpermeability, suppresses extravasation and leukocyte migration and preserves endothelial cell morphology.^{23, 24} In addition, it has also been demonstrated to have modulatory effects on the TNF- α mediated inflammatory pathways.²⁵ In order to mimic these findings, here we aimed to mimic the effects of cetirizine and aescin on reversing the morphology of endothelial cells that were induced by histamine or TNF- α

respectively. The effects of the change in morphology were assessed using immunostaining against actin and adherens junction (VE-cadherin) proteins.

5.2 Materials and Methods

5.2.1 Cell Culture and Seeding

Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured with endothelial growth media (PromoCell GmbH, ECGM-2, supplemented with 50U/ml Penicillin/Streptomycin (ThermoFisher, P/U)) in T75 culture flasks coated with 0.1mg/ml collagen I (Rat tail collagen I, ThermoFisher) in PBS (ThermoFisher). The cells were incubated at 37°C in humidified air with 5% CO₂. Flasks with confluent monolayers were either used for experiments or subcultured. HUVEC were kept in culture up to passage number 6. Prior to cell seeding, a 96 wells plate (Greiner) was coated with 0.1mg/ml collagen I for 30min at 37°C. After that the wells were washed with PBS once to remove non-bound collagen. This was followed by obtaining HUVEC from a confluent flask using 0.05% Trypsin-EDTA (ThermoFisher). Suspended in fresh ECGM-2, HUVEC were seeded at a density of 0.625×10^5 cells/cm² to each well.

In these wells, HUVEC were incubated for two days in ECGM-2 followed by changing the media to ECBM-2 (supplemented with 2% FBS and 1% P/S) for one day. Afterwards, cells were incubated either with ECBM-2 or ECBM-2 containing either cetirizine (3.1 µg/ml, in dPBS, Sigma Aldrich) or aescin (7.5 µg/ml in methanol, Sigma Aldrich) for one day prior to addition of TNF-α (1-100ng/ml in dPBS, PeproTech) and histamine (1-500 µM in dPBS, SigmaAldrich) the following day. For histamine exposure, cells were treated with histamine and/or cetirizine for 10, 20 or 30min at 37°C with 5% CO₂. For TNF-α exposure, cells were incubated with various concentrations of TNF-α in ECBM-2 for 24h and 48h. Cells were refreshed daily with fresh media, and throughout the whole experiment in wells containing cetirizine or aescin, cells were subjected to these drugs. All experiments were performed in triplicates.

5.2.2 Cell Staining

As a confirmation of cell morphology, cells were stained for actin filaments, nuclei and expression of specific cell adhesion markers. For that, cells were fixed with 4% formaldehyde (in PBS, ThermoFisher) for 10min at room temperature (RT). The fixative was washed away with PBS and the cells were incubated in permeabilization buffer (PB), which contains 0.1% Triton X-100 (Sigma Aldrich) and 10mg/ml bovine serum albumin in PBS for 15min at RT. This was followed by incubating the cells with goat anti-human VE-Cadherin IgG (0.5 µg/ml in PB, R&D Systems) for 2h at RT. Following incubation, the cells were rinsed three times with PBS and then washed three times with PBS for 10min at RT. Afterwards the cells were incubated for 1h at RT with donkey anti-goat IgG Alexa 546 (2.5 µg/ml, ThermoFisher), 6.25 µg/ml 4',6-Diamino-2-Pheylindole (DAPI, ThermoFisher) and 1 drop/ml Actin-Green (binds to actin filaments, ThermoFisher) in PB. After staining, cells were rinsed three times with PBS and then washed three times with PS for 10min RT.

The Cells were imaged with fluorescence microscopy using the EVOS FL2 Auto Imaging System (Life Technologies; RFP filter (ex 531/40 em 593/40) for VE-Cadherin, GFP filter (ex 470/22 em 510/42) for Actin-green, and DAPI filter (ex 357/44 em 447/60) for DAPI).

5.3 Results and Discussion

5.3.1 Effect of Histamine and Cetirizine on HUVEC

The role of inflammation in AMD pathogenesis has been well established and extensively reported in the literature.^{1, 9, 12} One of the main reasons for this relation is the presence of complement factors in drusen in AMD, activation of complement system and elevated levels of complement factors in AMD patients.²⁶ Two of these factors, C3a and C5a are also responsible for activating mast cells and further recruiting them to the site of complement activation.^{27, 28} Even though there are mast cells normally present in choroid, however, number and activity of these cells are elevated in AMD disease conditions in choroid.²⁹ These activated cells secrete histamine among many other pro-inflammatory factors which increases permeability of endothelial cells.¹⁸ Increase in permeability in this case, presumably have beneficial effects such as increased access of nutrients and oxygen to surrounding tissues, more traffic of leukocytes to the inflamed regions to improve tissue repair.^{8, 18, 19} On the other hand, continuous, lasting and imbalanced increase in tissue permeability will eventually lead to cell injury by hemorrhage and exacerbated immune response.^{1, 11, 12, 29}

Figure 5.1 Histamine exposure of HUVEC for 10min with or without the presence of cetirizine. HUVEC were either left untreated (UT) or treated with various concentrations of histamine (1, 10, 100, 500 μ M) for 10min. Following exposure, cells were immunostained for morphology with VE-cadherin (adherens junctions, red), actin (actin cytoskeleton, green), DAPI (nuclei, blue). Scale bar: 50 μ m.

In this part of the study, we aimed to simulate the disease conditions induced by the presence of histamine in the endothelial cell permeability. One of the indications of increased permeability could be seen by the change in cell morphology and cell-cell adhesion marker expression. In previous studies, histamine was shown to cause the rearrangement of actin

Figure 5.2 Histamine exposure of HUVEC for 20min with or without the presence of cetirizine. HUVEC were either left untreated (UT) or treated with various concentrations of histamine (1, 10, 100, 500 μ M) for 20min. Following exposure, cells were immunostained for morphology with VE-cadherin (adherens junctions, red), actin (actin cytoskeleton, green), DAPI (nuclei, blue). Scale bar: 50 μ m.

cytoskeleton and induced intercellular gap formation by disrupting adhesive properties of adherens junctions.

Decrease in the activity of adherens junctions was mediated via phosphorylation of tyrosine, which could be detected even after 1min of histamine exposure, and visible changes in adherens junctional staining were observed within 25min.³⁰ In contrary to these findings in other studies, in our experiments we did not observe such a difference in distribution of actin (Fig.5.1-3) nor did we detect intercellular gaps in the VE-cadherin expression. In addition, longer durations (30min) of histamine exposure also did not affect cell morphology significantly (Fig.5.3).

Inactivity of histamine on our cells might be due to the maturation of the junctional proteins, as earlier reports claim that the longer cells remain in confluency (48-72h) the less likely they were to be activated.³⁰

As none of the reports claim to expose the cells to histamine longer than 30min, this suggests that the activity of histamine might be difficult to maintain and be alleviated by intercellular repairing mechanisms. To prolong the effects, inclusion of serum factors might be useful, as it was reported to increase the permeability of endothelial cells³¹ suggesting that to see an effect in our experiments other factors might be necessary to include. This finding is indeed in line with the concept of para-inflammation in AMD pathophysiology, that in order to simulate disease conditions, prolonged exposures of histamine combined with patient serum should be combined to induce a long-lasting increase in permeability.

Figure 5.3 Histamine exposure of HUVEC for 30min with or without the presence of cetirizine. HUVEC were either left untreated (UT) or treated with various concentrations of histamine (1, 10, 100, 500 μ M) for 30min. Following exposure, cells were immunostained for morphology with VE-cadherin (adherens junctions, red), actin (actin cytoskeleton, green), DAPI (nuclei, blue). Scale bar: 50 μ m.

Moreover, along with including activated serum and its factors, incorporation of immune cells (*i.e.* leukocytes) which provide stable, continuous histamine to induce a morphological change in cells would be of great interest for a realistic model of AMD.

In this experimental setup, we also aimed to observe the effects of a common histamine antagonist, cetirizine. This compound was reported to be a fast-acting, highly selective antagonist of the H1- histamine receptor that endothelial cells possess. It binds with higher affinity to its receptor, and as a result histamine activity on endothelial cells is inhibited.²¹ Even though the compound was reported to be acting rapidly by typically beginning 20-60min and remaining active for at least 24h, we made sure there was always cetirizine available to cells throughout the experiment. In our experimental setup, we opted for 3.11 μ g/ml cetirizine concentration which was 10 times higher than the peak plasma concentration.³² According to our experiments, we did not observe any toxic effect of cetirizine on the cells as the cell number and morphology did not change at all in our control samples that did not contain any histamine (Fig 5.1-3, UT). However, since we did not observe a change in morphology with histamine exposure, we could not observe any visible change with cetirizine treatment.

5.3.2 Effect of TNF- α and Aescin on HUVEC

In addition to prolonged dysfunction of retinal tissues in AMD pathophysiology, recruited immune cells release proinflammatory factors such as TNF- α which exacerbates the sequence of events leading to photoreceptor degeneration. Released in the

microenvironment, TNF- α affects endothelial cell morphology and structure.³³ For instance, cells in monolayer become elongated and larger; actin fibers are oriented along the major axis of the cells.³³⁻³⁵ Considering the reported effects of TNF- α , our findings are in line with existing reports that there is a dose-dependent change in the morphology of cells in higher concentrations (Fig.5.4-5, 10-100ng/ml). The change in morphology was not visible in cells exposed to 1ng/ml TNF- α , however, it was clear in higher concentrations (10-100ng/ml). In addition, lower concentrations of TNF- α (1ng/ml) induced a comparable change in morphology of cells with longer exposure times (48h, Fig.5.5) which simulates the prolonged effects of para-inflammatory disease conditions.

Figure 5.4 TNF- α exposure of HUVEC for 24h with or without the presence of aescin. HUVEC were either left untreated (UT) or treated with various concentrations of TNF- α (1, 10, 100ng/ml) for 24h. Following exposure, cells were immunostained for morphology with VE-cadherin (adherens junctions, red), actin (actin cytoskeleton, green), DAPI (nuclei, blue). Scale bar: 50 μ m.

On the other hand, the protective effects of aescin, which was added to the cells 24h prior to TNF- α exposure could not be observed as it seemed to be toxic for cells and the ones remaining were elongated even in non-TNF- α treated samples which indicates stress. Here, methanol was used as a solvent for aescin, and its toxicity should be well tolerated by the cells in the used dosages as reported previously for human cancer cell lines.³⁶

Some reports mention that the aescin concentrations used in our study (7.5 μ g/ml) did not significantly reduce the cell viability on HUVEC.³⁷ However, according to other reports this concentration induced significant toxicity on HUVEC.²³ As there is no consensus on which concentration is optimal for cell viability, the exact dosage of aescin remains to be elaborated. Also, from a para-inflammatory point-of-view it is important to investigate the longer durations of low dosages of aescin.

The exact mechanism of action of aescin is not clear, however, it has been shown to reduce the secretion of TNF- α and IL-1 β in macrophages, decreasing the blood flow by increasing

Figure 5.5 *TNF- α exposure of HUVEC for 48h with or without the presence of aescin.* HUVEC were either left untreated (UT) or treated with various concentrations of TNF- α (1, 10, 100ng/ml) for 48h. Following exposure, cells were immunostained for morphology with VE-cadherin (adherens junctions, red), actin (actin cytoskeleton, green), DAPI (nuclei, blue). Scale bar: 50 μ m.

venous tension, and reducing capillary permeability antagonizing the activity of bradykinin, a known peptide that promotes inflammation.²⁴

5.4 Conclusion and Future Outlook

The role of para-inflammation in the development of AMD has been getting more and more attention in the literature over the decades. Therefore, developing simplified treatments that tackle the disrupted activity of the immune system on retinal homeostasis and its prolonged detrimental effects on dysfunction of retinal tissues are of great importance. In addition there is a chance these treatments will replace the existing treatments which are expensive and often stressful for patients.

In our experiments, we used histamine and TNF- α in order to induce a disease phenotype in HUVEC using morphology as a readout for increased permeability. Even though we were able to induce this with TNF- α , we did not observe changes with histamine exposure. One of the factors might be due to the treatment of histamine not being stable enough to observe a distinct change that is homogeneous throughout the whole monolayer. For histamine exposure, since we could not observe a difference in the cell morphology, we could not observe any healing effects of cetirizine treatment either. For TNF- α exposure on the other hand, aescin was proved to be toxic for the cells in the used dosage.

There are various challenges in creating a realistic *in vitro* disease model for AMD due to the disease being multifactorial and because para-inflammatory processes take decades to have an effect on patients. In addition, these processes possibly require the inclusion of serum and activated immune cells into the microenvironment. Therefore a representative model of AMD would require prolonged exposure of cells to disease stimuli with

combination of patient serums and activated immune cells. Furthermore, patient stem cell-derived endothelial cells can also be incorporated to the model to capture the genetic component of the disease and generate a more realistic model of AMD. Together, these factors would provide proper experimental conditions for mimicking AMD and to develop novel treatments.

Using organ-on-a-chip (OOC) devices mentioned previously, it is possible to mimic the native microenvironment of a blood vessel. This has been demonstrated by previous reports, that immune cells (THP-1 monocytes or neutrophils isolated from fresh human blood) can be incorporated inside the channels lined with endothelial cells.^{38, 39} As the channel is perfused with cell media, immune cells can be recirculated as well.³⁹ Extravasation of immune cells can be tracked in these reports, as well as cytokine profile secreted from cells in culture can be analyzed by drawing cell medium. To induce a disease state, cells are commonly stimulated using proinflammatory cytokines (interleukin-13, interferon- γ) or lipopolysaccharide.^{40, 41} However, to induce a chronic stress disorder such as AMD, where initiation of symptoms require continuous low level stress for many decades, it would be favorable to use human plasma based on patients, albeit availability of this might be a challenge. Patient-specific plasma not only contains disease stimuli present in the patient, but also might provide favorable media composition for different cells co-culture environment.

If such a more inclusive disease model is developed, it may be very favorable to use induced pluripotent stem cells derived cells, as it will mimic a very realistic microenvironment of patient's own respective tissue. Reprogrammed stem cells which are originally collected from patients can be differentiated into endothelial and immune cells, and be incorporated into OOC devices. Such a realistic model would provide proper experimental conditions for mimicking AMD and to develop novel treatments.

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6

Summary and Future Outlook

In this chapter, a summary of the thesis chapters, recommendations for future work and an outlook are provided.

6.1 Summary

This thesis provides a model of the outer blood retinal barrier in an organ-on-a-chip (OOC) device for investigation of age related macular degeneration (AMD). Mimicking pathophysiology of AMD is in particular challenging, as visible symptoms could be experienced after many decades of continuous stress on retinal tissues. Wet-AMD, which is the form of AMD this project aimed to tackle here, is characterized by the choroidal vessel ingrowth into the retina, which eventually leads to photoreceptor degeneration due to leakage from these new ingrown vessels. Although vessel growth is the characteristic trait of wet-AMD, permeability increase is the main reason behind vision loss. Therefore, here we focused on this rather earlier event in disease pathology. Experimental treatment that was mentioned in Chapter 1 and 5 is not against the vessel ingrowth but aimed at normalizing the vessel permeability so that natural homeostasis agents could work towards reducing cellular stress.

Chapter 2 describes different methods of measuring permeability in conventional and OOC platforms which are transepithelial/endothelial electric resistance, paracellular diffusive transport of tracer molecules, hydraulic conductivity of water across a cellular monolayer. It addresses advantages and disadvantages of these methods in different applications as well as lists an overview of different models with their respective readout for barrier function assessment. It is essential to understand these limitations when designing a modelling platform for which a readout might not be suitable.

Chapter 3 describes an OOC model developed in-house, that employs a microvessel lined with an endothelial cell monolayer, an open-top culture chamber inhabited by a retinal pigment epithelial cells and a polyester based membrane separating two culture compartments. A healthy co-culture is maintained using a gravity driven medium flow in channels. This platform is suitable for applying two clinically relevant readouts to investigate the vascular dysfunction and structure. Vascular dysfunction measurement is based on fluorescein angiography in which fluorescently labeled dextran is perfused and its permeability through endothelial barrier is measured. Capabilities of optical coherence tomography is demonstrated in terms of visualizing microvascular networks. In our platform, the majority of vessels resemble capillary-like vessels, proving this platform can be used to monitor changes in AMD. In addition, one of the disease stimuli, accumulation of reactive oxygen species, is used to induce dose and exposure dependent vascular dysfunction. Inclusion of clinically relevant readouts in complex *in vitro* models is essential for bridging the gap between fundamental research and its potential application in clinic.

Chapter 4 studies membranes made of collagen I hydrogel that are integrated into aforementioned devices. Vitrification-based fabrication of these membranes results in micrometer-thick membranes that are biocompatible. Bruch's membrane is partially degraded by matrix remodeling enzymes so that choroidal vessels are able to infiltrate the retina. This diseased state is modeled using enzymatic degradation based on collagenase II treatment. It is demonstrated that the enzymatic treatment decreases the permeability of membranes significantly, however it destabilizes the membranes which is demonstrated by bursting of membranes when treated longer and with higher enzyme concentrations. Furthermore, this chapter provides an injection mold design which is used to incorporate membranes into devices with consistent thickness and prevents labor-intensive conventional fabrication method using PDMS mortars.

Chapter 5 describes the importance of para-inflammation in the pathophysiology of AMD as well as the need for new treatments against inflammation. To this end, two over-the-counter drugs, aescin (anti-inflammatory) and cetirizine (anti-histaminic) were tested in their ability to revert the possible damaging effects of TNF- α and histamine on 2D monolayers of endothelial cells in culture plates. TNF- α is observed in making a change in morphology of the cells in a dose-dependent manner, whereas histamine was not observed as having such effects. As a result, effects of cetirizine in the treatment of histamine exposed cells could not be observed, and in the case of aescin, it was proved to be toxic to the cells. In this case, stability of molecules as well as the homogeneity of the exposure is debatable. Even though the effects cannot be observed, this chapter provides an elaborate discussion on how a realistic *in vitro* AMD disease model would look for the simulation of AMD-on-a-chip technology.

6.2 Conclusion

Overall, the aim of this thesis to develop a model of the oBRB for the investigation of AMD was achieved. Translation from OOC research to clinic was facilitated by making use of clinically relevant readouts to evaluate the disease state of cells in our model. As an extension of the project, we demonstrated the use of realistic biological membranes in our model. These membranes were fully characterized in terms of permeability and response to enzymatic treatment, which will pave the way for modelling one of the components of oBRB, Bruch's membrane, and its role in choroidal neovascularization. The effects of inflammation and experimental treatment could not be observed as there might be a need for low continuous doses of these stimuli in a more realistic model of the oBRB. In the future, a more realistic model of the oBRB for AMD disease modeling could be achieved by using induced pluripotent stem cell derived cells from patients, by inclusion of patient serum and by inclusion of local immune cells. Achieving this is all certainly feasible within the coming years.

The OOC oBRB modelling platform has received significant attention from a broad community and blossomed into further collaborations. For instance, a recent grant was awarded to Dr. Andries van der Meer for incorporating stem cell-derived cells into these models in a consortium composed of partners from universities (University of Twente, Radboud University Medical Center, Leiden University Medical Center) and commercial bodies (Boehringer Ingelheim GmbH).

6.3 Future Outlook

Complete reduction of animals as models as well as adoption of OOCs by clinicians for disease modelling is only possible by building better models that recapitulate the microenvironment of human tissues as realistically as possible. There are certain aspects of our model which are paramount to building a better model of the oBRB in AMD research. These are listed below and should act as starting points for the next in line of this research:

- Longer maintenance of cellular barriers: as AMD is a chronic, slow-paced disorder in which the damage accumulates over time, it is essential for cells to be stable in culture for longer periods of time. Even though a first step could be achieved by culturing the

devices on a rocking platform mentioned in Chapter 3, this platform only provides a bidirectional perfusion, which is not physiologic. Therefore, it is important to employ feeding platforms that provide automated, unidirectional perfusion. Media should also be recirculated not only to be cost-effective but also to build up the amount of disease stimuli within the system.

- **More realistic cell types:** Even though very close, using human umbilical vein endothelial cells (HUVEC) and human immortalized retinal pigment epithelial cells (RPE) do not reflect the native tissues of the retina. These cells were useful in building the first generation of the model with relevant readouts. However, there is a need to improve the platform with patient-specific stem cell derived co-cultures of RPE and choroidal endothelial cells in order to be able to induce AMD disease pathology. These cells are not only realistic representations of the tissues of the oBRB but they also contain genetic predispositions of AMD patients, which would not be possible to integrate otherwise.
- **Media incompatibility:** As the number of cell types increase in devices, media compatibility becomes an issue against having a tight cellular barrier. In our current model, RPE and HUVEC could be cultured together in endothelial growth media, however, other specialized cell types might not have that flexibility. In this case, first ensuring tight cell barrier with one type of cell followed by the addition of the other cell type might minimize mixing of media to another culture area.
- **Realistic membranes:** Even though we report a realistic biologically responsive collagen I membrane, Bruch's membrane (BM) is a multi-layered, specialized membrane that is highly complex, consisting of various extracellular matrix proteins. Full recapitulation of BM may not be realistically possible, as the exact composition is not entirely known. Maintaining a longer culturing time might enable cells to deposit their own respective BM components, however the required timing of this event as well as a proper readout of such a structure are ambiguous and may require another research project on its own.
- **In addition to using patient-derived cells to integrate genetic predispositions in a realistic model,** it is also essential to incorporate disease stimuli actively present in patient's body. For instance, in the case of incorporating inflammatory molecules, it is important to obtain plasma from patients, and mixed with cell media to possibly maintain a longer culture, but also to induce AMD pathology specific to patients.

7

Appendix

Supplementary Information and Figures

7.1 Supplementary Information for Chapter 3

7.1.1 Materials and Methods

Effect of Reactive Oxygen Species and Permeability Assay

For oxidative stress experiments, HUVECs were seeded at a density of $0.75 \cdot 10^5$ cells/cm² on a culture wells plate (Gibco) which was coated with 0.1mg/ml collagen I. HUVECs were incubated for a day in endothelial growth medium (EGM-2: EBM-2 with EGM-2 SingleQuots, Lonza) followed by changing media to EBM-2 (supplemented with 2% FBS and 1% P/S). The next day, cells were incubated in various concentrations of H₂O₂ (50-1000μM) dissolved in EBM-2 (with 2% FBS and 1% P/S) for 1, 2 or 5 days. Every day, the medium was refreshed with freshly made H₂O₂ solutions.

Following treatment, cells were counted using images of nuclei which were stained with DAPI. First, images were converted to 8-bit and applied threshold in order to develop a contrast between images and background using Fiji software.³⁸ After that, each image was converted to binary to apply watershed on the particles, where multiple nuclei were divided into single nuclei. Then, particles (pixel size: 10- infinity, circularity: 0.00-1.00, excluded on edges) were counted.

For permeability experiments, HUVECs were seeded at a density of $1 \cdot 10^5$ cells/cm² on a Transwell insert (Corning) which was coated with 0.1mg/ml collagen I. HUVECs were incubated for a day in EGM-2. After that, media was changed to EBM-2 (with 2% FBS and 1% P/S). The following day, cells were incubated in various concentrations of H₂O₂ (Sigma Aldrich, Germany) dissolved in EBM-2 (with 2% FBS, 1% P/S). (50-1000μM) for 1, 2 or 5 days. The levels of medium in both Transwell compartments were equalized to avoid pressure-driven fluid flow. After exposure to H₂O₂, the medium in the insert was replaced with EBM-2 containing fluorescently labelled dextran (FITC-Dextran, 40 kDa, fluorescein label (ex/em: 494/521), ThermoFisher) at a concentration of 15μg/ml, whereas the medium in the bottom well was replaced with fresh EBM-2. After that, from the beginning of the experiment, a sample of 50μL was collected from the bottom well every 30min and the levels were normalized by adding EBM-2 to the bottom compartment. These samples were read by a plate reader (Victor3, Perkin Elmer). Using a standard curve, fluorescence values were matched with concentrations. The permeability (P_{cell}) of HUVEC was calculated by:

$$P_{total} = \frac{dC}{dt} \frac{Volume_{well}}{C_i Area_{well}} \quad (7.1)$$

$$\frac{1}{P_{cell}} = \frac{1}{P_{total}} - \frac{1}{P_{empty}} \quad (7.2)$$

With P_{total} the permeability in cm/s, C_i the initial dextran concentration in μg/ml. $Area_{well}$ and $Volume_{well}$ are the dimensions of the bottom compartment in cm² and dC/dt was the change in the concentration (μg/ml·s) (Equation 1).³⁹ To calculate the permeability coefficient of the cells, the coefficient of an empty insert was subtracted from the cell containing insert (Equation 2).

7.1.2 Results

HUVEC Morphology and Permeability under H₂O₂ Exposure

HUVECs in culture well plates were exposed to various concentrations of H₂O₂ for 1, 2 and 5 days. While low concentrations did not result in considerable cell loss as compared to untreated cells, higher concentrations significantly decreased the cell number (Suppl.Fig.7.1.1A). Using these concentrations, changes in barrier integrity of HUVECs were tested in a Transwell system as a mean of dextran diffusion. Here it is worth noting that passage of dextran was only possible through the intercellular spacing of the cells, and the Transwell membrane was not a limiting factor in the diffusion of the tracer. Upon quantification of permeability, we could observe a dose and exposure dependent response to H₂O₂ (Suppl.Fig.7.1.1B) which was consistent with the morphology of HUVECs (Suppl.Fig.7.1.1C). Continuous exposure with high concentrations of H₂O₂ resulted in a barrier close to an empty insert, thus not represented.

Supplementary Figure 7.1.1: Assessment of HUVEC morphology and barrier function under H₂O₂ exposure. (A) Cell number in response to H₂O₂ were calculated (results were normalized to untreated). Error bars indicate standard error of the mean. (B) In addition, permeability of HUVEC was assessed using a Transwell system. Compared to untreated cells (UT), a dose dependent response (UT vs. condition) in permeability was detected in higher H₂O₂ concentrations and exposure. Values equal or worse than an empty insert were not represented (Denoted by hashes). (C) Dose dependent response to H₂O₂ was evident in morphology of HUVEC. Red: Phalloidin (actin filaments), blue: DAPI (nuclei). n=3. Scale bar: 400µm.

Supplementary Figure 7.1.2: Overview of the OCT setup. (A) Schematic overview of the OCT setup. Light travels from the source to the sample. Spectrometer calculates the distance based on the comparison with piezo driven mirror. BS: Beam splitter, L: Lens, PDM: piezo driven mirror, S: Sample, MS: motorized stage, SMF: single mode fiber. (B) Schematic overview of the OOC device with inset showing the cross-section view of the channel. (C) Phase contrast image of the chip with a fibrin matrix, with arrow heads showing the formation of vascular network branches. Scale bar: 100μm.

Supplementary Figure 7.1.3: Illustration of localized enhanced dye accumulation in the microvessel. Exemplary image represents a device with HUVEC and ARPE-19 co-culture. Speculative dye accumulation after removal of labeled dextran was shown by red arrows. Contrast in raw images were enhanced using a lookup table. Microvessel and channel borders are indicated by red and white lines respectively. Scale bar: 200μm

Supplementary Figure 7.1.4: HUVEC morphology upon H_2O_2 exposure inside microvessels. Representative images of cells fluorescently stained for nuclei (DAPI; blue) and actin filaments (green). Scale bar: $100\mu m$.

Supplementary Figure 7.1.5: Dye accumulation in the epithelial culture chamber in devices with endothelial monocultures. Representative image of a device with HUVEC mono-cultures inside the microvessel after treatment with $800\mu M H_2O_2$ for 5 days. Dye accumulation in the epithelial culture chamber (black line) before (i) and following FITC-dextran perfusion (ii) is shown. Red arrows indicate the dye accumulation in the culture chamber. Contrast in raw image is enhanced using the lookup table on the right. Microvessel and channel borders are indicated by red and white lines respectively. Scale bar: $200\mu m$.

7.2 Supplemental Information for Chapter 4

7.2.1 Results

Supplementary Figure 7.2.2: Overview of the permeability assay. Permeability of membranes was measured by means of fluorescein diffusion. (A) On-chip permeability assay first starts with filling the culture chamber with PBS (i). Second, fluorescein sodium salt diluted in PBS was pipetted into the microchannel (ii), and every 15min, 5 μ l of sample was collected from the culture chamber (iii). The levels were normalized by adding PBS afterwards (iv). In order to avoid heterogeneity in dye concentration along the microchannel, dye transferred from one inlet to the other after every sampling. **(B)** Permeability assay in transwells were used for comparison. (From left to right) First, fluid levels were normalized in insert (containing fluorescein) and bottom well (containing PBS). Afterwards, permeability assay was performed by collecting samples every 15min and normalizing the levels subsequently by adding PBS. **(C)** From both systems, the samples were collected and amount of fluorescein were measured using a plate reader. The fluorescent intensity profile was matched to concentration to calculate the permeability of the membranes.

Supplementary Figure 7.2.2: High concentrations of collagenase treatment may cause ruptures on collagen membranes. (A) OOC device schematics where membrane was integrated. (B-C) Two separate ARPE-19 seeded membranes stained for nuclei (DAPI, blue) were treated with 120U/ml collagenase II 3 days after cell seeding. Upon treatment membranes (red dashed lines) were detached from the culture chamber borders (white dashed lines). In addition, unstable membranes were punctured (white arrow heads) or left fragments (red arrows). Scale bars: 300µm.

Supplementary Figure 7.2.3: Morphology and confluency of ARPE-19 cultured on collagen membranes in the OOC device. ARPE-19 stained for nuclei (DAPI, blue, left) and actin cytoskeleton (phalloidin, green, right) reveals a homogeneous distribution of a healthy population of ARPE-19. Scale bar: 50µm.

Supplementary Figure 7.2.4: SEM image of a single and multi layered collagen I membrane. (A) A fibrillar, and ~2µm thick membrane was obtained after the fabrication process. Scale bar: 1µm. (B) A multi-layered membrane containing 4 and 3mg/ml collagen I from top to bottom respectively. Scale bar: 10µm.

Supplementary Figure 7.2.5: Devices integrating collagen I membranes can potentially be fabricated using injection molding design which eliminates the labor intensive assembly of devices. (A) Schematic overview of the fabrication of devices using injection molding. (A-left) Empty assembled mold prior to PDMS injection. (A-middle) assembled mold after PDMS injection. (A-right) Resulting PDMS devices following bake of PDMS. (B-left) Schematic overview of the final assembled device. (B- right) Resulting devices contain membranes free of PDMS where both channels intersect. Black PDMS was utilized to provide clarity and better illustration of the clean membrane area. Scale bar: 1mm.

Supplementary Figure 7.2.6: ARPE-19 cell grown on collagen I coated glass cover slips. These cells express their respective cell-cell adhesion markers (ZO-1) indicating a healthy population. Scale bar: 50μm.

8

Samenvatting

Özet

Scientific Output

Funding and Contributions

Acknowledgements

8.1 Samenvatting

In dit proefschrift wordt een model gepresenteerd van de buitenste bloed-retinabarière in een zogenaamd *organ-on-a-chip* (*OOC*) systeem voor onderzoek naar leeftijdsgebonden maculadegeneratie (LMD). Het nabootsen van de pathofysiologie van LMD is een uitdaging, met name omdat symptomen pas opgemerkt worden na decennia van voortdurende achteruitgang van het weefsel. Dit project was voornamelijk gericht op natte LMD, dat wordt gekarakteriseerd door ingroei van vaten uit het vaatvlies in de retina. Dit leidt uiteindelijk tot degeneratie van fotoreceptoren door lekkage van deze ingegroeide vaten. Hoewel vaatgroei karakteristiek is voor natte LMD, is toenemende permeabiliteit de voornaamste reden voor verminderd visus. Daarom is ervoor gekozen om eerst deze eerste stap van de degeneratie te onderzoeken. Experimentele behandelingen, zoals besproken in Hoofdstuk 1 en 5, zijn niet gericht op ingroei van vaten, maar op het herstellen van de permeabiliteit van de vaten zodat natuurlijke homeostasemechanismen cellulaire stress tegen kunnen gaan.

Hoofdstuk 2 beschrijft verschillende methoden om permeabiliteit te meten in conventionele en *OOC* platforms. Dit zijn transepitheel/endotheel elektrische weerstand, paracellulair diffuus transport van tracer moleculen en hydraulische weerstand van cellulaire monolagen. Voor- en nadelen van deze methoden worden beschouwd en er wordt een overzicht gegeven van de verschillende modellen en hun respectievelijke uitlezing en de beoordeling van de barrièrefunctie. Het is belangrijk om deze limitaties te begrijpen bij het ontwerpen van een modelplatform.

In Hoofdstuk 3 wordt een *OOC* model gepresenteerd dat hier ontwikkeld is, waarbij een microvat wordt gebruikt dat omlijnd is met een endotheelcelmonolaag, in een open kweekomgeving. Deze bevat retinale pigmentepitheelcellen en een polyester membraan dat twee compartimenten scheidt. Een co-cultuur wordt in leven gehouden door hydrostatisch aangedreven toevoer van medium in microkanalen. Dit platform is geschikt voor het uitlezen van twee klinisch relevante parameters om de structuur en het disfunctioneren van de vaten te bestuderen. Vatdisfunctie wordt gemeten met fluoresceïne-angiografie waarin fluorescent gelabeld dextran wordt geperfuseerd en waarbij de permeabiliteit van de endotheelbarrière wordt gemeten. Er wordt aangetoond dat optische coherentietomografie kan worden ingezet om microvasculaire netwerken te visualiseren. In dit platform lijkt een meerderheid van de vaten op haarvaatjes, hetgeen bewijst dat op deze manier veranderingen in LMD kunnen worden gevolgd. Daarnaast wordt een van de ziekte-inductie stimuli (openeenhoping van reactief zuurstof) ingezet om vatdisfunctie te initiëren die afhankelijk is van dosis en blootstelling. De inclusie van klinisch relevante parameters in complexe *in vitro* modellen is essentieel om fundamenteel onderzoek ooit in de kliniek toe te kunnen passen.

In Hoofdstuk 4 worden membranen bestudeerd gemaakt van een collageen-I-hydrogel die geïntegreerd worden in de voorgenoemde systemen. Fabricage gebaseerd op vitrificatie van deze membranen geeft biocompatibele membranen van enkele micrometers dik. Bruch's membraan wordt deels gedegradieerd door hermodelleringsenzymen zodat choroïdale vaten de retina kunnen binnendringen. Dit stadium van LMD wordt gemodelleerd met enzymatische degeneratie door collagenase II. Het wordt aangetoond dat deze enzymatische behandeling de permeabiliteit van de membranen aanzienlijk verlaagt. Echter wordt ook het membraan gedestabiliseerd, wat zich uit in het barsten van de membranen bij langere

behandelingen of hogere enzymconcentraties. Er wordt ook een spuitgietontwerp gedeeld in dit hoofdstuk, waarmee membranen met constante dikte kunnen worden opgenomen in het systeem. Dit maakt de inefficiënte fabricage met PDMS-cement overbodig.

In hoofdstuk 5 wordt het belang van para-inflammatie in de pathofysiologie van LMD uitgelegd, alsmede de behoefte aan nieuwe behandelingen tegen inflammatie. Hiertoe worden twee vrij verkrijgbare medicijnen getest; aescin, een ontstekingsremmer, en cetirizine, een antihistamine. Er wordt gekeken naar het mogelijk teruggedraaien van beschadigende effecten van TNF- α en histamine op 2D endotheelcelmonolagen in kweekplaten. Van TNF- α werd opgemerkt dat het de morfologie dosisafhankelijk beïnvloedt, maar histamine niet. Daardoor kon het effect van cetirizine niet worden aangetoond. Aescin bleek giftig, en de stabiliteit van de moleculen en de homogeniteit van de blootstelling is discutabel. Er konden geen effecten worden aangetoond, maar er wordt wel een uitgebreide discussie gevoerd in dit hoofdstuk over het ideale model van LMD-stimulatie op een chip.

8.2 Özet

Bu tez, yaşa bağı maküler dejenerasyonun (YMD) araştırılması için mikroakışkan çipler üzerinde oluşturulan dış kan retina bariyerinin (dKRB) bir modelini sunmaktadır. YMD'nin hastalık patofizyolojisini modellemek özellikle zordur, çünkü retina dokularında ancak onlarca yıllık süregelen stresin ardından hastalarda görünür semptomlar yaşanabilir. Bu projede ele alınan YMD'nin en yaygın formu olan ıslak-YMD, koroidal kılcal damarların retinaya doğru büyümesi ile karakterize edilir. Retinaya doğru büyüyen damarların içindekileri retinanın içine sızdırmalarıyla fotoreseptörler dejenerasyona uğrarlar. Bunun sonucu olarak hastalar merkezi görüşlerini kaybederler. Damarların retinaya doğru büyümesi ıslak YMD'nin karakteristik özelliği olarak kabul görmesine rağmen, bu damarların geçirgenliklerindeki artış görme kaybının arkasındaki ana nedendir. Bu nedenle, burada hastalık patofizyolojisini modellerken bu daha erken gerçekleşen olguya odaklandık. Bölüm 1 ve 5'te bahsedilen deneysel tedavi damarın büyümesine karşı değil, aksine geçirgenliğini düzenleyerek ederek tedaviyi etmeyi amaçlamaktadır. Damarların geçirgenliklerinin azalmasıyla, vücuttaki homeostasiyi koruyan sistemler hücresel stresi azaltmaya yönelik çalışabilir.

Bölüm 2 hücreler tarafından oluşturulan bariyerlerin geçirgenliğinin ölçülebilmesi için kullanılan farklı metodları tanımlamaktadır (transepitel / endotel elektrik direnci, işaretlenmiş moleküllerin hücreler arasından difüzyon yoluyla taşınması, ve hücresel tabakanın hidrolik iletkenliğinin ölçümü). Bu yöntemlerin uygulamalardaki avantaj ve dezavantajlarına değinmenin yanı sıra, son olarak kullanılan metodlar ve farklı organ modelleri listelenmiştir. Her bir yöntemin limitleri anlaşılırsa uygun bir modelleme platformu tasarlanabilir.

Bölüm 3, laboratuvar bünyesinde dizayn edilen ve geliştirilen bir organ çipini betimlemektedir. Bu çip, mikro damar içinde endotel hücre tabakası kaplı olan bir mikrokanal, ortasında polyesterden imal edilmiş yarı-geçirgen bir zar, ve bu zarın üzerinde konuşlanmış retinal pigment epitel hücre tabakasından oluşmaktadır. Mikrokannallardaki hücrelerin beslenmesi yerçekiminin yardımıyla gerçekleşmektedir, böylece hücrelerin doğru biçimde beslenmesi sağlanmaktadır. Bu platform, vasküler disfonksiyon ve yapılarının araştırmak için klinik olarak benzer iki adet ölçüm metoduyla çalışmaktadır.

Vasküler disfonksiyon ölçümü floresanla işaretli dekstran sölüsyonunun mikrokannallarda perfüze edilmesiyle yapılırken, öte yandan mikrovasküler ağıın görselleştirilmesi optik koherens tomografi ile tespit edilmiştir. Platformumuzda tespit edilen vasküler yapıların çoğunluğu makuladaki kılcal damarların boyutlarında olması, bu platformun YMD modellemesi için kullanılabileceğine kanıt niteliğindedir. Ek olarak, bu çalışmada vasküler disfonksiyonu sağlamak için reaktif oksijen türlerinin birikimini, doza ve süreye bağılı olarak indüklendiğini kanıtladı. Klinikte kullanılan ölçüm metodlarının bu gibi platformlara dahil edilmesi bilimsel araştırmalar ile klinik araştırmaların arasındaki mesafenin giderilmesi açısından çok önemlidir.

Bölüm 4, daha önce bahsedilen çip düzeneğine laboratuvar ortamında üretilen tip 1 kolajenden yapılmış zarları incelemektedir. Bu zarların üretimi vitrifikasyon adı verilen bir süreçle yapılmaktadır, ve ortaya çıkan zarlar mikrometre kalınlığında olmaktadır. Daha önceki bölümlerde belirtildiği üzere dKRB bariyerinde epitel ve endotel hücrelerini ayıran kısımda özelleşmiş bağ doku proteinlerinden oluşan karmaşık bir Bruch's zarı vardır. Bu zar hastalık durumlarında matrix modelleme enzimleri tarafından kısmi olarak yeniden düzenlendiği takdirde bu endotel hücrelerinin retinaya büyümesine aracı olmaktadır. Bu özelliği nedeniyle, zarın bu durumu modelde kullanılmıştır. Zarda meydana gelen değişiklikleri collagenase II enzimi kullanarak gerçekleştirdik. Enzimatik işlemle zarın geçirgenliğinin önemli ölçüde önce azaldığı, yüksek konsantrasyonda ve uzun süre enzime maruz kalmasıyla zarın kararlılığının bozulduğu tespit edilmiştir. Ayrıca bu bölüm, zarların tutarlı kalınlığa sahip cihazlara dahil etmek için kullanılan bir enjeksiyon kalıbının tasarımını da içermektedir.

Son olarak Bölüm 5, YMD'nin patofizyolojisinde para-inflamasyonun önemini ve inflamasyona karşı yeni tedavilere olan ihtiyacı açıklamaktadır. Bu amaçla, iki yaygın kullanılan ilaç, aescin (anti-inflamatuvar) ve setirizin (anti-histaminik), TNF- α ve histamin 2D hücre kültürlerindeki endotel hücrelerinin üzerindeki olası zararlı etkilerini iyileştirmeleri açısından test edildi. TNF- α , doza bağılı bir şekilde hücrelerin morfolojisinde bir değişiklik yaparken gözlenirken, histaminin bu tür etkilere sahip olduğu deneylerimizde gözlenmemiştir. Sonuç olarak, setirizinin histamine maruz kalan hücrelerin tedavisindeki etkileri gözlenemedi ve aescin durumunda ise hücreler için toksik olduğu kanıtlandı. Bu durumda, moleküllerin stabilitesi ve maruziyetin homojenliği tartışmalıdır. Etkiler gözlemlenemese bile, bu bölüm gerçekçi bir modelin bir çip üzerinde YMD simülasyonunu nasıl arayacağına dair ayrıntılı bir tartışma sunmaktadır.

8.3 Scientific Output

8.3.1 Peer-reviewed journal articles

Y.B. Arik, W. Buijsman, J. Loessberg-Zahl, C. Cuartas-Vélez, C. Veenstra, S. Logtenberg, A.M. Grobbink, P. Bergveld, G. Gagliardi, A.I. den Hollander, N. Bosschaart, A. van den Berg, R. Passier, A.D. van der Meer. “Microfluidic Organ-on-a-Chip Model of the Outer Blood-Retinal Barrier with Clinically Relevant Read-Outs for Tissue Permeability and Vascular Structure” *Lab on a Chip* (2020) 21, 272-283

Y.B. Arik*, A. de sa Vivas*, D. Laarveld, N. van Laar, J. Gemser, T. Visscher, A. van den Berg, R. Passier, A.D. van der Meer. “Collagen I Based Enzymatically Degradable Membranes for Organ-on-a-Chip Barrier Models”. *ACS Biomater. Sci. Eng.* (2021)

*equal contribution

P. Das, A.D. van der Meer, A. de sa Vivas, **Y.B. Arik**, J.C. Remigy, J.F. Lahitte, R.G. Lammertink and P. Bacchin. “Tunable Microstructured Membranes in Organs-on-Chips to Monitor Transendothelial Hydraulic Resistance” *Tissue Engineering Part A* (2019), 25(23-24), 1635-1645.

W.F. Quirós-Solano, N. Gaio, O.M.J.A. Stassen, **Y.B. Arik**, C. Silvestri, N.C.A. van Engeland, A.D. van der Meer, R. Passier, C.M. Sahlgren, C.V.C. Bouten, A. van den Berg, R. Dekker, P.M. Sarro. “Microfabricated Tuneable and Transferable Porous PDMS Membranes for Organs-on-Chips.” *Scientific Reports*, (2018) 8(1), 1-11.

Y.B. Arik, M.W. van der Helm, M. Odijk, L.I. Segerink, R. Passier, A. van den Berg, A.D. van der Meer. “Barriers-on-chips: Measurement of Barrier Function of Tissues in Organs-on-Chips.” *Biomicrofluidics* (2018), 12(4), 042218.

8.3.2 Conference Contributions

Proceedings

W.F. Quirós-Solano, N. Gaio, C. Silvestri, **Y.B. Arik**, O.M.J.A. Stassen, A.D. van der Meer, C.V.C. Bouten, A. van den Berg, R. Dekker, P.M. Sarro. “A Novel Method to Transfer Porous PDMS Membranes for High Throughput Organ-on-a-Chip and Lab-on-Chip Assembly”. *IEEE Micro Electro Mechanical Systems* (2018), 318-321.

Y.B. Arik, R. Matsui, E. Sarajlic, Y. Takayama, B. Boom, E. Berenschot, S. Le Gac, N.R. Tas, H. Fujita. “Ultra-Thin Nanochannel-Based Liquid TEM Cell for EELS Analysis and High Resolution Imaging.” *IEEE 29th International Conference on Micro Electro Mechanical Systems* (2016), 804-807.

Oral Presentations

Y.B. Arik, P. Bergveld, A.I. den Hollander, E. de Jong, A. van den Berg, R. Passier, A.D. van der Meer. “Unravelling Macular Degeneration: An Organ-on-a-Chip Model to Study Outer Retinal Layers of the Human Eye.” *International Organ-on-a-Chip Symposium*, Lausanne, Switzerland. (2017).

Y.B. Arik, P. Bergveld, A. van den Berg, R. Passier, A.D. van der Meer. “Unravelling Macular Degeneration: An Organ-on-a-Chip Model to Study Outer Retinal Layers of the Human Eye.” *Micro Nano Conference*, Amsterdam, The Netherlands (2016).

Poster Presentations

Y.B. Arik, W. Buijsman, C. Veenstra, S. Logtenberg, A.M. Grobbink, P. Bergveld, G. Gagliardi, A.I. den Hollander, N. Bosschaart, A. van den Berg, R. Passier, A.D. van der Meer. “Microfluidic Organ-on-a-chip Model of the Outer Retinal Barrier with Readouts for Permeability and Vascular Structure” *European Organ-on-a-Chip Symposium* (2020).
*held online.

Y.B. Arik, W. Buijsman, C. Veenstra, S. Logtenberg, A.M. Grobbink, P. Bergveld, G. Gagliardi, A.I. den Hollander, N. Bosschaart, A. van den Berg, R. Passier, A.D. van der Meer. “Development of a Microfluidic Organ-on-a-chip Model of the Outer Retinal Barrier for Studying Vascular Dysfunction in Age-related Macular Degeneration” *European Organ-on-a-Chip Symposium*, Graz, Austria (2019).

Y.B. Arik, A.M. Grobbink, P. Bergveld, E. de Jong, A.I. den Hollander, A. van den Berg, R. Passier, A.D. van der Meer. “Unravelling Macular Degeneration: An Organ-on-a-chip Model of the Human Retina”. *European Organ-on-a-Chip Symposium*, Stuttgart, Germany (2018).

Y.B. Arik, P. Bergveld, T. Radstake, J. Kuiper, A.I. den Hollander, E. de Jong, A. van den Berg, R. Passier, A.D. van der Meer. “Unravelling Macular Degeneration: An Organ-on-a-chip Model of the Human Retina”. *International Organ-on-a-Chip Symposium*, Enschede, The Netherlands (2016).

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8.4.1 Funding

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8.4.2 Contributions

Chapter 1 & 6 were written by Yusuf Arik with input from Andries van der Meer and Piet Bergveld.

Chapter 2 was based on a review article in which Yusuf Arik and Andries van der Meer wrote the manuscript with critical feedback on the contents by Marinke van der Helm, Mathieu Odijk, Loes I. Segerink, Robert Passier, and Albert van den Berg.

Chapter 3 was based on a publication in which Yusuf Arik and Andries van der Meer designed the experiments and critically analyzed data. Yusuf Arik and Wesley Buijsman performed chip design and developed the microvessel patterning protocol. Joshua Loessberg-Zahl and Yusuf Arik developed and performed the fibrin gel patterning for OCT measurements. Anne Grobbink and Yusuf Arik performed and analyzed endothelial seeding and morphology experiments. Carlos Cuartas-Vélez, Colin Veenstra, Sander Logtenberg, Nienke Bosschaart developed OCT setup, performed OCT analysis. Yusuf Arik and Andries van der Meer wrote the manuscript. Piet Bergveld, Anneke I. den Hollander, Giuliana Gagliardi, Robert Passier, and Albert van den Berg advised on key experimental design criteria, critically advised on data analysis and reviewed the manuscript.

Chapter 4 was based on a publication Yusuf Arik co-authored with Aisen de sa Vivas. In this work, Yusuf Arik, Aisen de sa Vivas, and Andries van der Meer designed the experiments and critically analyzed data. Yusuf Arik and Aisen de sa Vivas performed and optimized both chip designs. Daphne Laarveld, Neri Van Laar, Jesse Gemser, Thomas Visscher fabricated collagen membranes, maintained cell cultures on membranes and performed immunofluorescent stainings. Daphne Laarveld and Thomas Visscher performed the permeability measurements on non-treated and enzymatically treated membranes. Robert Passier, Albert van den Berg critically advised on data and reviewed the manuscript.

Chapter 5 is a proof of concept chapter on which Yusuf Arik performed and optimized the histamine and TNF- α exposure on cells and optimized the staining protocols. It was written by Yusuf Arik with input from Piet Bergveld and Andries van der Meer.

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